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A Study of the Wahl Peptase Method

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A STUDY OF THE WAHL PEPTASE METHOD

BY

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PREFACE

An interest was first aroused in this problem by Mr. Lawrence Ehrnst of the Froedtert Malting Company who has been interested for some time in devising a method for determining the peptic power of malt. Through the cooperation of Dr. J. R. Koch, the author's major professor, a study of the Wahl Peptase Test was made possible.

Anyone who attempts the study of enzymes is confronted with the various factors inhibiting enzyme action. Also, the seemingly contradictory results of different investigators is somewhat confusing. This may be attributed to the difficulty of repeating identical experimental conditions, for enzymes are very sensitive to outside factors which affect enzyme action.

This work on the analysis of the Wahl method is but a part of a series of investigations to be carried on in an attempt to obtain a satisfactory test for the peptic power of malt. A complete study of the Wahl method was impossible because of the limited time.

The writer wishes to thank John R. Koch, Ph. D., for his helpful suggestions concerning viscosity determinations. The writer is also indebted to the Froedtert Malting Company for the use of their laboratories, and to Lawrence E. Ehrnst and George Yakish for their encouragement and kind assistance.

INTRODUCTION AND HISTORY

Unfortunately we have, as yet, no test at our disposal which will inform us as to the amount of proteolytic power in malt which could compare in simplicity and rapidity with the starch-iodine reaction. Concerning the proteins, the laboratory methods are involved and lengthy, as are also the methods used for determining proteolytic power.

Very little work has been done in this country on the proteolytic power of malt infusions, although considerable work from different angles has been carried on in Germany. The Wahl Peptase method, developed by the Wahl Institute, Chicago, Illinois, has been the subject of study and analysis by the writer. The time element, one of the important factors in laboratory routine work, is unfavorable in the Wahl method because of the great amount of time required to carry out the test.

A review of the work done recently (mostly in Germany) on the proteolytic effect of malt infusions and worts has been given by P. Kolbach and H. Simon.[1]

Adler's Work:

1. Used as a substrate-edestin and gelatin and also a mixture

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[1]Brewers Technical Review, (The Proteolytic Power of Malt Infusions and Worts), Siebel Publishing Co., Chicago, Ill., Nov. 1936, p. 351.

of albumin modification products known as Witte-Peptone.

2. Edestin and Witte-Peptone were noticeably affected by malt infusions. Gelatin was more or less unaffected.

3. Degree of proteolysis was determined by Formol-titration.

Lundin's Work:

1. Worked with gelatin and Witte-Peptone.

2. Gelatin showed a liquefaction. Witte-Peptone showed an increase of Formol-titration nitrogen.

3. Found that a substrate of acid-albumin (egg-white treated with acid) was unmodified in his experiments.

Mill's and Linderström-Lang's Work:

1. Found that acid albumin is not attacked by proteases of malt.

2. Modifications were observed in gelatin, edestin, egg-albumin-peptone and leucylglycine.

3. Concluded that there were two proteolytic enzymes.

   (a) A Proteinase--which acts on gelatin, edestin, and egg-albumin-peptone.[2]

   (b) A Peptidase--which splits the dipeptide leucyglycine.

4. Edestin was found to be very suitable for the determination of the effect of proteinase.

   (a) Kjeldahl Method was used.

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[2]Ibid., p. 351.

Lüers' and Malson's Work:

1. Used gelatin and leucylglycine to determine the proteinase and peptidase present in malt infusions and enzyme preparations.
2. Proteolysis action determined by means of acidimetric titration in alcohol solution (increase of carboxyl groups from splitting of peptide-linkage).

Hopkins' and Burns' Work:

1. Determined Proteolysis by amount of protein not coagulating when the solution was boiled.
2. Witte-Peptone was used as a substrate.
3. Observed breaking of peptide-links by Formol-titration.

Idoux's Work:

1. Used barley flour as substrate.
2. Enzymes (proteolytic) in barley flour are destroyed on drying at 105° C. for 4 hours.
3. Concluded that barley flour contains some proteins which must be modified in the process of brewing.

In Experiments:

1. Edestin selected as best substrate.
2. $\frac{N}{10}$HCl used to dissolve substrate.
3. Enzyme solution--enzyme plus distilled water to a volume of 49 cc. One drop of chloroform used to prevent bacterial

action.

Preparation of Malt Infusion:

1. 75 g. of malt made up to 495 g. of solution.
2. Mashed for one hour at 20° C. and then stored overnight at 3° C. It is then filtered through a folded filter at room temperature.
3. 300 cc. of filtrate was treated with 0.3 cc. of $CHCl_3$, shaken, and stored at 3° C. until needed.
4. Action of proteinase on edestin--was stopped by NaOH. NaOH neutralizes the HCl. A pH of 5.6 was attained.
5. Heated on reflux for ten minutes--precipitating unmodified edestin. Water bath was found more suitable.
6. 10 cc. of filtrate--used for determining the nitrogen by the Kjeldahl method.
7. Edestin--optimum pH at 3.9--4.1. It dissolves sparsely in buffer solutions. Edestin dissolves in HCl and is precipitated by buffer solutions.
8. Varying reaction periods were used (3 to 48 hours) at 45° C.[3]

Method Used for the Determination of the Proteolytic Power of Malt Infusion and Wort.

1. 1 g. of edestin dissolved in 6 cc. of $\frac{N}{10}$HCl.

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[3]Kolbach and Simon, Brewers Technical Review, Nov. 1936, p. 352.

2. Enough malt infusion added to result in 5-18 mg. of modified nitrogen per 10 cc. of filtrate.
3. Distilled water added to obtain 49 cc. volume and then one drop of $CHCl_3$.
4. Warmed for 6 minutes in water bath at 35° C. with gentle shaking.
5. After thorough stirring the solution is heated for 6 hours at 35° C.
6. Cooled and treated with 6 cc. of $\frac{N}{10}$NaOH and boiled on a reflux for ten minutes.
7. Cooled, filtered, and 10 cc. of filtrate is used in determining nitrogen content by Kjeldahl method.

As there is no assurance of identical composition of commercial edestin, a new diagram or graph must be made for each edestin run.[4] The same may be said for the different gelatines, all of which are supposedly pure but their compositions vary greatly as the writer has found by experiments with two different gelatines--both of which were pure.

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[4]Ibid., p. 352.

I

THEORIES REGARDING ENZYMES AND ENZYME ACTION

According to Hawk and Bergeim, we may define an enzyme as an "organic catalyst which is elaborated by an animal or vegetable cell and whose activity is entirely independent of any of the life processes of such a cell. From this definition the enzyme zymase elaborated by the yeast cell is entirely comparable to the enzyme pepain elaborated by the cells of the atomach mucosa. One is derived from a vegetable cell, the other from an animal cell, yet the activity of neither is dependent upon the integrity of the cell."[5]

In regard to the chemical nature of enzymes we find two distinct schools. Perhaps the greatest number of those who have investigated enzymes are advocates of Willstätter's school. Their contention is that enzymes are composed of a specific active group and a colloidal bearer or carrier and that the specific group binds the enzyme to the carrier. The other concept concerning the nature of enzymes is held by Fodor and his associates. They assert that the enzymes exist in peculiar colloidal systems and depend upon their colloidal nature for their activity.

Enzymes may be classified in accordance with the type of action they cause and accelerate. Hydrolytic enzymes pro-

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[5]Hawk & Bergeim, "Practical Physiological Chemistry", 1931, P. Blakiston's Son & Co., Philadelphia, p. 234.

duce hydrolysis, oxidases and peroxidases effect oxidizations, reductases bring about reduction, and protein-coagulating enzymes produce coagulation of proteins. The hydrolytic enzymes are subdivided in accordance with the class of compounds they act upon. A terminology is used in which the ending --lytic or --clastic replaces the final syllable of the compound or class (Greek: lysis, loosing; klastos, broken in pieces). Proteolytic or proteoclastic enzymes catalyse the hydrolysis of proteins, amylolytic or amyloclastic enzymes catalyse the hydrolysis of starches, and lipolytic or lipoclastic enzymes catalyse the hydrolysis of fats.[6]

The specificity of enzymes in regard to the substrate or substance acted upon is a well known fact. Emil Fischer investigated this problem of specificity extensively in connection with the fermentation of sugars and reached the conclusion that enzymes with the possible exception of certain oxidases, can act only upon such substances as have a specific stereo-isomeric relationship to themselves. He contends that an interrelationship must exist between the enzyme and substrate, such as the key has to the lock, or otherwise the reaction does not occur. This is shown by the specificity enzymes show in that some act on fats, while others act on

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[6] Cameron, "Textbook of Biochemistry," Macmillan Co., 1935, New York, p. 12.

carbohydrates or proteins.[7]

In all enzyme reactions there are definite factors which effect the rate at which the substrate changes. These factors are:

1. Concentration of the substrate.
2. Concentration of the enzyme.
3. Temperature.
4. Reaction or hydrogen-ion concentration of the medium.
5. Light and other radiations.
6. Electrolytes.
7. Inhibiting agents or poisons.
8. Products of the reaction.

In the majority of enzymatic reactions it has been found that the speed of the reactions increases with increasing concentration of the substrate; then it remains constant for a considerable addition in concentration, and finally decreases in very concentrated solutions of the substrate.[8]

The velocity of a reaction has been found to vary with the amount of enzyme used. Schutz, in studying the action of Pepsin on protein, observed that the quantities of protein hydrolyzed in equal periods of time by different amounts of

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[7]Hawk & Bergeim, Practical Physiological Chemistry, pp. 238-239.
[8]Bodansky, Meyer, Introduction to Physiological Chemistry, John Wiley & Sons, New York, 1934, p. 138.

pepsin are proportional to the quantity of enzyme used.[9] From this a relationship known as the Schutz-Borissov rule has been expressed thus:

$$X = K\sqrt{aEt}$$

where X equals mass of protein transformed; E, the concentration of the enzyme; the time, t; the initial concentration of the substrate, a.[10]

Generally a rise in the temperature increases the rapidity of a chemical reaction. All enzymatic reactions have an optimum temperature and also a temperature above which the destruction of the enzyme takes place. This optimum temperature varies for different enzymes but for most enzymes it is between 37° and 53° C. Enzymes of plant origin are known to have a higher optimum temperature than those from animal tissue. Papain and Bromelin, which are proteolytic enzymes found in plants have a temperature optimum about 60°. Some of the milk-coagulating enzymes found in plants are known to have a considerably higher optimum, approximately 80-85° C.[11]

Acids and alkalies have much to do with enzyme action. Some enzymes act only in acid solution while others are inactivated in alkaline medium. Pepsin acts only in an acid med-

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[9] Cameron, Textbook of Biochemistry, p. 27.
[10] Bodansky, Meyer, Introduction to Physiological Chemistry, p. 139.
[11] Ibid., p. 142-143.

ium and is inactive in an alkaline solution, whereas trypsin does not act in the presence of free acid but is active in a neutral or alkaline solution. Northrup says that the determining factor in the digestion of proteins by pepsin is the quantity of ionized protein in solution. The type or nature of an acid is not as important as might be thought. It has been shown by Northrup that at equivalent hydrogen-ion concentration the nature of the acid used does not change the rate of digestion. He found that the rate of peptic digestion of gelatin, egg albumin, casein, and edestin is the same in solutions of nitric, hydrochloric, sulfuric, oxalic, citric, and phosphoric acids. Acetic acid differs somewhat in that it diminishes the rate of digestion of all proteins except gelatin. This may be attributed to the effect of the acetic acid on the protein rather than on the enzyme.[12]

Light and other radiations are generally not injurious to enzymes--although in the presence of oxygen light will usually activate enzymes to some extent. Electrolytes of inorganic salts may either increase of slow up the speed of enzymatic reactions. Heavy metals, antiseptics, the halogens, protein precipitants, and flourides all exert an inhibiting effect on enzymes.[13]

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[12]Ibid., p. 144-45.
[13]Ibid., p. 148-151.

II

THE DECOMPOSITION AND HYDROLYSIS OF PROTEINS

In the decomposition of proteins, many products are formed, of which the amino acids are considered the the most important. Among the decomposition products are the proteases, peptones, peptides, ammonia, carbon dioxide, hydrogen sulphide, and the amino acids.

The proteoses which are formed first give a purple biuret reaction although a light purple. The proteoses are soluble in water and are precipitated with ammonium sulphate. Upon further hydrolysis peptones are formed. The peptones are more soluble in water than the proteoses. They give a pink biuret reaction and are not precipitated by ammonium sulphate. As hydrolysis proceeds a mixture of amino acids is produced and the biuret reaction is no longer given.[14]

These biuret reactions alone show a distinct difference in the decomposition products of the proteins.

Physically, the hydrolysis of proteins consists in a breaking down of the large, colloidal, non-diffusible complexes into a series of fragments in which the colloidal character becomes less and less pronounced, until, finally, only the simple crystalloidal and diffusible amino acids

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[14]Cameron, Textbook of Biochemistry, pp. 108-109.

remain.[15]

When a protein has an equal number of positive and negative electrical charges it is said to have reached the "iso-electric point."[16] Each protein has a definite pH value at which the protein exists practically in a non-ionized state. This is the iso-electric point of the protein. Thus if we add acid to a protein solution at its iso-electric point, increasing the hydrogen-ion concentration, acid salts are formed and ionized. Likewise if the protein solution is made alkaline, metal proteinates are formed and ionize. At its iso-electric point a protein is least soluble, and advantage is taken of this condition in the preparations of pure proteins from their solution.[17]

Bacterial Decomposition of the Proteins.

Proteins may be decomposed by bacterial action as well as by hydrolysis. It is from the putrefactive decomposition of the proteins that the most toxic substances are produced. The amino acids of the proteins set free by the intestinal enzymes are physiologically inert. The bacteria, however, like the cells of the body, have the power of tearing these amino acids to pieces and some of the products are very toxic.[18]

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[15]Hawk & Bergeim, Practical Physiological Chemistry, p. 84.
[16]Read, Industrial Chemistry, John Wiley & Son, Inc., New York, 1933, p. 418.
[17]Cameron, Textbook of Biochemistry, p. 136.
[18]Mathews, Physiological Chemistry, p. 455.

It is known that the bacteria have the power of deamidizing the amino acids, setting free ammonia and leaving the fatty acid. Besides the deamidizing reaction, is one involving the removal of a carboxyl group (decarboxylation), probably due to an enzyme carboxylase, present in the bacteria. Reduction, due to a reductase, and reactions of hydrolysis as well as of oxidization are also believed to occur.[19]

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[19]Bodansky, Meyer, Introduction to Physiological Chemistry, p. 204.

III

METHODS OF MEASURING ENZYME ACTIVITY

As enzymes are rarely available in a pure state, the only means we have of measuring the strength or activity of an enzyme preparation is to determine the amount of substrate which a given amount of enzyme will hydrolyze under carefully controlled conditiohs.

In measuring an enzyme action on a substrate, we use definite amounts of both enzyme and substrate and allow the reaction to proceed for a definite length of time. The amount of substrate unchanged or changed may be measured directly, or a physical property which changes in the course of the reaction may be measured.

In an analysis to determine the extent of protein modification in malt, the principal steps involved are: 1. the total protein, both in the malt and the wort is determined according to the regular Kjeldahl method;--2. the soluble protein in the wort is divided into co-agulable and non-coagulable parts; --3. the extent to which the complex proteins in the soluble and non-coagulable fraction are modified or broken down into simpler proteins is measured by determining one group of the decomposition products. This is done by means of the "Formol-titration" which shows the amount of the final and most simple

substances resulting from proteolytic action namely the polypeptides, peptides, and amino-acids.

Various other methods have been used in determining enzyme activity. A commonly used procedure for the determination of peptic activity is the Neirenstein and Schiff modification of Mett's method. Small glass tubes, filled with coagulated egg albumin, are introduced into small flasks containing a definite amount of gastric juice, adjusted to approximately 0.05N HCl. Digestion occurs at both ends of the tube; the portion digested at each end is measured and used as a basis for calculating the peptic activity.[20]

When gelatin is used as the substrate the enzyme action may be measured by the changing viscosity of the solution.

Various types of viscometers have been used by different investigators. Among these may be mentioned the Modified Bloom Viscometer and the Hoppler Viscometer. Hoppler found that the viscosity varies with concentration and that the unfermentable matter has a higher viscosity.[21] Lüers and Löther employed the Hoppler viscometer in determining the breakdown of gelatin by papain and other proteolytic plant enzymes. Lüers and Löther state that enzyme reactions can be followed conveniently by the use of a viscometer.[22]

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[20] Hawk & Bergeim, Introduction to Physiological Chemistry, p. 176.

[21] Wochschr, Brau, 51, 161-165 & 169-171, (1934).

[22] Wochschr, Brau, 52, 49-51, (1935).

IV

MATERIALS AND METHODS USED

The Wahl Peptase Test:

Essentially the Wahl Peptase test is a means of determining the peptic power or activity of malt. Tubes containing gelatin of the same quantity but with different concentration of enzyme, are heated for 4 hours at 41° C. Likewise tubes containing a like amount of gelatin plus 5 cc. of malt solution are reacted under the same conditions. After 4 hours of heating at 41° C. the tubes are placed in a 21° C. water bath and the time of solidification of each tube is noted. Thus, from the gelatin-enzyme tube that corresponds in solidification time to the malt-gelatin tubes, the amount of enzyme present or the peptic power of the malt may be calculated. Thus if the malt-gelatin tubes solidify in 15 minutes and one of the enzyme-gelatin tubes also solidifies in 15 minutes, the concentration of enzyme in the malt will be equivalent to that of the enzyme in the gelatin tube which solidified in the same length of time.

Preparation of Bacterial Lactic Acid.

The Bacterial Lactic Acid was prepared in the following manner.

1. One part of crushed malt to four parts of water.

2. The mixture is inoculated with the lactic acid ferment, containing the Bacillus Delbrucki.

3. Heat for two hours at 45° C.

4. The liquid is then drawn off from the mash.

5. The liquid is again heated at 45° C. for 72 hours.

It was found that the acidity of the lactic acid formed was between 1.5 and 2% acid. A bacterial lactic acid solution which was 1.5% acid was found to have a normality of 0.167 and a pH of 3.17. The Wahl test calls for a 2% bacterial lactic acid solution. A slight amount of C. P. lactic acid can be added in case the bacterial acid formed is considered too weak. The writer's lactic acid solutions were slightly less than 2% acid, but were not thought weak enough to effect or change the reaction.

It was found more convenient to make a large amount of the bacterial acid at a time rather than small amounts, due to the time involved. Also much liquid is absorbed by the mash and in making small amounts of the acid there is quite a loss of liquid. It was found that 1500 grams of crushed malt and 6000 cc. of water would make approximately one gallon of the bacterial acid.

The medium used for propagation of the culture was a Glucose-Yeast-Water-Agar. The culture should be changed once

a month.

Of great importance in the understanding of fermentation is the work on zymase by Harden and Young. By filtration through a layer of gelatin under a pressure of 50 atmospheres, pressed yeast juice can be divided into a filtrate and a residue, which are separately inactive towards sugar but produce fermentation when again mixed.[23] The filtrate or substance in the dialystate resists boiling and undergoes hydrolytic decomposition and destruction by enzymes (lipases) of the yeast juice. But the zymase itself, which does not go through the filter is destroyed when heated and is probably a protein substance, being attacked by the proteinases or proteases of the yeast juice.[24] Alcoholic fermentation by means of pressed yeast juice is activated by the addition of a phosphate.[25]

Harden and Young have made the discovery that during the time of enhanced fermentation, the amount of carbon dioxide produced exceeds that which would have been formed in the absence of phosphate by a quantity exactly equivalent to the phosphate added--CO_2 : $R_2'HPO_4$.[26]

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[23]Euler, General Chemistry of the Enzymes, (from Proceedings of the Physiol. Soc., Nov. 12, 1904, see Journal of Physiol., 1904, 32, i; Proc. Roy. Soc. B., 1906, 77, 405), John Wiley & Sons, Inc., New York, 1912, p. 53.
[24]Euler, General Chemistry of the Enzymes, p. 53.
[25]Ibid., p. 54.
[26]Ibid., p. 54 (from Proc. Roy. Soc. B., 77, 405, 1906).

A zymase is defined as the intracellular enzyme of yeast by which alcoholic fermentation is produced.[27]

An active preparation of Bacillus Delbrucki was made by Buchner and Meisenheimer, which, however, only gave rise to small quantities of dl-lactic acid. The preparation was made as follows: the organism was cultivated at 40-45° C. in a wort made from malt and rye.[28]

The bacteria were later separated by means of a centrifuge and dried on a porous file. The mass was then put into 15-20 times its weight of acetone, with which it was ground for 10-15 minutes, the bacteria being pumped dry, washed with ether and dried in a vacuum.[29]

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[27]Definition given in the American Illustrated Medical Dictionary, W. B. Saunders Co., Philadelphia, 17th Edition, 1936.

[28]Euler, General Chemistry of Enzymes, p. 58 (from Chem. Ber., 1930, 36, 635, Lieb. Ann. 1906, 349, 125).

[29]Ibid., p. 58.

Preparation of Gelatin Solutions.

A 10% gelatin solution was made by putting 10 g. of gelatin in a 100 cc. volumetric flask and diluting with distilled water to 100 cc. It was found that if a small amount of water was put in the flask before adding the gelatin--liquefaction could be attained more easily upon heating and the gelatin would not adhere to the bottom of the flask.

The gelatin was dissolved in the solution by immersing the flask containing the gelatin solution in a water bath at 41° C. for a few minutes. After the gelatin was completely dissolved it was transferred to test tubes by means of a pipette. Ten cc. of the 10% gelatin solution was placed in each test tube.

Type of Gelatin Used.

Two different gelatins were used in the Wahl test. The one found more suited was the Wahl isinglass, a type of gelatin which was obtained from the Wahl Institute in Chicago, Illinois. A second gelatin was also used but it did not prove as satisfactory as the Wahl isinglass. The Wahl gelatin solidified more quickly than the second type of gelatin, although both gelatines were supposedly pure preparations.

Gelatins are very difficult to distinguish from one another, their behavior being very similiar to reagents. Gelatins of different origin, however, have undoubtedly a different

composition, the nitrogen content being variable. Some observers have thought that the different gelatines have different specific rotations and may be so distinguished.[30]

Hide pieces, fleshings, and animal bones, comprise the principal raw material for the manufacture of gelatin and glue; there are also minor sources of raw material which will yield a satisfactory gelatin. The bladders of some fish (e.g., the sturgeon) are washed, purified and dried with rolling to make "isinglass," a form of natural gelatin in which the original fibrous structure is retained.[31]

Gelatin is usually classified as belonging to the Scleroproteins (Greek: --skleros, hard) class of proteins. The members of the class do not occur in plants. One of their distinctive features is their marked insolubility. Gelatin is generally included in this group, more or less for convenience, though it is extremely soluble, and should be, perhaps, called a derived protein instead of a true protein.[32]

In the amino acid composition of gelatin, cystine, tryptophane, and valine are missing, and Histidine and Tyrosine are present in very minute amounts. It is interesting to know that the price of gelatin is based on the amount of volume it will give, and this, of course, is based on its jellying

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[30]Bennett, Hugh G., "Animal Proteins, Baillieri, Tindall & Cox, London, 1921, p. 5.
[31]Ibid., p. 228.
[32]Cameron, Textbook of Biochemistry, p. 130.

strength. Some gelatines are weaker in jellying strength and, therefore, are cheaper than those with a higher jellying strength. Oftentimes the higher jellying strength gelatin does not require as much in a mixture as the other.[33]

Edestin has been used by many investigators as a substrate instead of gelatin. Edestin differs from gelatin in that it is not soluble in water, though it is soluble in dilute solutions of sodium chloride. Upon heating edestin again differs from gelatin in that it coagulates.[34]

In the two different kinds of gelatin which were used by the writer, one was found to solidify in 5 minutes (Wahl gelatin) and the other took 7 minutes before solidifying. This shows a difference in the composition of the two gelatines although both were pure gelatin.

Enzymes Used, and Their Properties.

The enzyme used was Wahl's standard peptase which was obtained from the Wahl Institute in Chicago, Illinois; caroid was also used in the tests but the results obtained from its use were not uniform, like those of the Wahl peptase.

The enzyme solution is made in the following concentration--1/10 gram to 400 cc. of bacterial lactic acid, so that

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[33]Excerpt from a letter received from W. C. Bittel of the Knox Gelatine Co., Jamestown, N. Y.

[34]Cameron, Textbook of Biochemistry, p. 105.

1 ml. of the bacterial lactic acid contains 1/4 milligram of active concentrated enzyme. The strength of the enzyme should be such so that 5 ml. of it just will completely proteolyze and destroy the jellifying power of the 10% solution of gelatin when digested for 4 hours at 41° C.

The "caroid" used is a commercial preparation and name given to the proteolytic enzyme papain. Enzymes from vegetable sources are much different in their properties than those derived from animal sources. Caroid shows some difference to pepsin in that it acts in acid, alkaline, or neutral solutions, while pepsin is active only in acid solutions.

Caroid is made from papain, which is the juice of the papaya (Carica papaya), containing the proteolytic enzyme papain, which is also called vegetable pepsin.[35] Papain can be heated at 70° C. for 1/2 hour without a perceptible change in enzymatic activity. It is destroyed at 82-83° C. The activity of papain is greatly increased by the addition of HCN and H_2S.[36]

Peptase.

During germination of the grains of cereals, the cereals secrete, besides amylase, other active substances, among them

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[35]Exerpt from literature obtained from the American Ferment Company, Buffalo, N. York.

[36]Waksman & Davison, Enzymes, Williams & Wilkins Co., Baltimore, 1926, p. 224.

peptase, which transforms albuminoid substances into amides.[37]

Proteninases is the name given to those enzymes which break down the true proteins. The decomposition appears to proceed as far as the albumoses and peptones. The proteniases rarely occur alone in the organs of plants where proteins are decomposed; they are generally accompanied by peptases, which corresponds with the erepsins of the animal body.[38] Euler defines peptases as those enzymes which decompose albumoses, peptones and polypeptides into amino-acids.[39] The peptases are found in the seeds of lupins, rape, peas, and maize.[40]

Properties of Pepsin.

Asher has shown that pepsin is harmfully affected by iron salts and arsenic salts.[41] It was reported by Grützner that peptic digestion is retarded by the presence of alkali sulphate.

Inorganic colloids (gold, platinum, silver, copper, arsenic, bismuth, mercury) do not accelerate pepsin, but when highly concentrated they seem to inhibit it.[42]

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[37]Effront & Prescott, Enzymes & Their Applications, John Wiley & S ons, New York, 1902, p. 148.
[38]Euler, General Chemistry of the Enzymes, pp. 38-39.
[39]Ibid., p. 40.
[40]Ibid., p. 41 (from Vines, Annals of Botany, 20, 113, 1906).
[41]Ibid., p. 117
[42]Ibid., p. 118.

In the presence of chloroform or toluene, fermentation is interrupted, although zymase itself is not injured by chloroform--as has been shown by experiments with press yeast-juice. Also, yeast cells invert cane sugar with the same rapidity with or without the presence of chloroform or toluene.[43]

In the activation of gelatin hydrolysis by papain the influence of Fe^{++} is very noticeable but ascorbic acid has no effect. This activation does not occur with highly purified papain. It is due to the action of an inhibitory substance designated X.[44]

Preparation of the Malt Solutions.

The malt solutions are made up in the ratio of one part of finely ground malt to five parts of 2% bacterial lactic acid. After the acid and malt have been mixed, the solution is heated for one half hour at 41° C. and is stirred several times during the heating. After heating for one half hour the solution is filtered and 5 cc. of the filtrate is added to 10 cc. of the 10% gelatin solution. The tubes are then placed in a 41° C. water bath for 4 hours, and at the end of the 4 hours the tubes are then put in a 21° C. water bath and the time of solidification of each tube is noted.

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[43]Euler, General Chemistry of the Enzymes, pp. 120-121.
[44]Ernst Maschmann, Z, Physiol. Chem., 228, 141-86, 1934.

Several different types of malt were used--as the peptic power of malt is quite variable. By comparing the times of solidification of the various malt-gelatin tubes with those containing the enzyme--the amount of enzymatic or peptic power of the malts can be determined.

In the Wahl Peptase Method it is evident that the bacteria of the lactic acid have some influence on the malt solution. In contrast to this, Kolbach and Simon used chloroform in the ratio of 1:1000 to improve the stability of their malt infusions. They found that the proteinase was slightly damaged by the $CHCl_3$ but if stored at 3° C. the malt could be kept for 3 days without loss in proteolytic power.[45]

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[45]Brewers Technical Review, (The Proteolytic Power of Malt Infusions and Worts), Siebel Publishing Co., Chicago, Ill., Dec. 1936, p. 393.

Hawk and Bergeim have given a table of the proteases as follows:[46]

Name and Class	Distribution	Substrate	End-products
Proteases		Proteins	
I. Coagulases		Proteins in Solution	
A. Rennin (Gastric)	Gastric juice	Casein	Paracasein
B. Thrombin	Blood	Fibrinogen	Fibrin
II. Pepsin (acid protease)	Gastric juice	Proteins	Proteoses and Peptones
III. Trypsin (alkali protease)	Pancreatic juice	Proteins	Proteoses, peptones, and peptides.
IV. Vegetable proteases			
A. Bromelin	Pineapple	Proteins	Proteoses, peptones, etc.
B. Papain	Pawpaw	Proteins	Proteoses, peptones, etc.
Phytase	Rice, bran, liver, blood	Phytin	Inosital and Phosphoric acid
Glycerophosphatase	Intestinal mucoaa, etc.	Glycerophosphotic acid	Phosphoric acid and glyceral
Hexosephosphatase	Intestinal mucosa, bone, etc.	Hexosephosphoric acid.	Phosphoric acid and hexose

[46] Hawk & Bergeim, Introduction to Physiol. Chem., p. 236.

V

EXPERIMENTAL PROCEDURE AND DATA

Procedure for the Wahl Peptase Test.

The procedure used for the peptic-enzyme-gelatine tubes is as follows:

Malt-Gelatine tubes (peptic enzyme in malt).

1. Add 5 parts of 2% bacterial lactic acid to 1 part of finely ground malt.
2. Stir well and keep at 41° C. for ½ hour.
3. Stir several times during period.
4. Filter solution.
5. Add 5 cc. of the malt filtrate to 10 ml. of the 10% gelatine solution.

Enzyme-Gelatine tubes (enzyme was Wahl's peptase).

The following are added to five test tubes containing 10 ml. of the 10% gelatine.[47]

To #1 test tube add 5 ml. of Wahl's peptase.[47]

To #2 test tube add 4 ml. of Wahl's peptase plus 1 ml. of lactic acid.[47]

To #3 test tube add 3 ml. of Wahl's peptase plus 2 ml. of lactic acid.

To #4 test tube add 2 ml. of Wahl's peptase plus 3 ml. of lactic acid.

To #5 test tube add 1 ml. of Wahl's peptase plus 4 ml. of lactic acid.

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[47] The methods used for preparing the lactic acid, 10% gelatine, and the Wahl peptase solutions are given on pages 16, 20, and 22 respectively.

The tubes are heated in a water bath for 4 hours at 41° C. At the end of this time the tubes are placed in a 21° C. water bath and the time of solidification of each tube is noted. The peptic strength of the malt may be easily found by noticeing which Enzyme-Gelatine tube has a solidification time similiar to that of the malt tubes. In this way it is possible to find the amount of pepsin contained in the malt, in terms of a standard pepsin whose activity is known.

From the concentration of the Wahl peptic enzyme it is evident that there is $\frac{1}{4}$ mg. of enzyme (peptic) in 1 ml. Therefore #1 tube equals $1\frac{1}{4}$ mg. enzyme (peptic).

#2 " " 1 mg. " "

#3 " " $\frac{3}{4}$ mg. " "

#4 " " $\frac{1}{2}$ mg. " "

#5 " " $\frac{1}{4}$ mg. " "

	Solidifies
10 ml. gelatine plus 5 ml. of lactic acid---	6 min.
10 ml. gelatine plus 5 ml. of dist. water---	5 min.

#1 tube---$1\frac{1}{4}$ mg. (Wahl's Peptase) ----------	60 min.
#2 tube---1 mg. " " ----------	35 min.
#3 tube---$\frac{3}{4}$ mg. " " ----------	25 min.
#4 tube---$\frac{1}{2}$ mg. " " ----------	15 min.
#5 tube---$\frac{1}{4}$ mg. " " ----------	12 min.

Thus, if a tube containing the malt filtrate solidifies in 15 minutes, it is equivalent in peptic strength to ½ mg. of Wahl's standard peptase.

The solidification times (on previous page) given for the five tubes containing the enzyme are those found by Wahl. The writer was able to substantiate these solidifying times by numerous experiments. However, in some cases the time varied slightly and this seemed to indicate that this solidification method is not as sensitive and accurate as might be desired.

The following table shows the solidification times of five different malts.

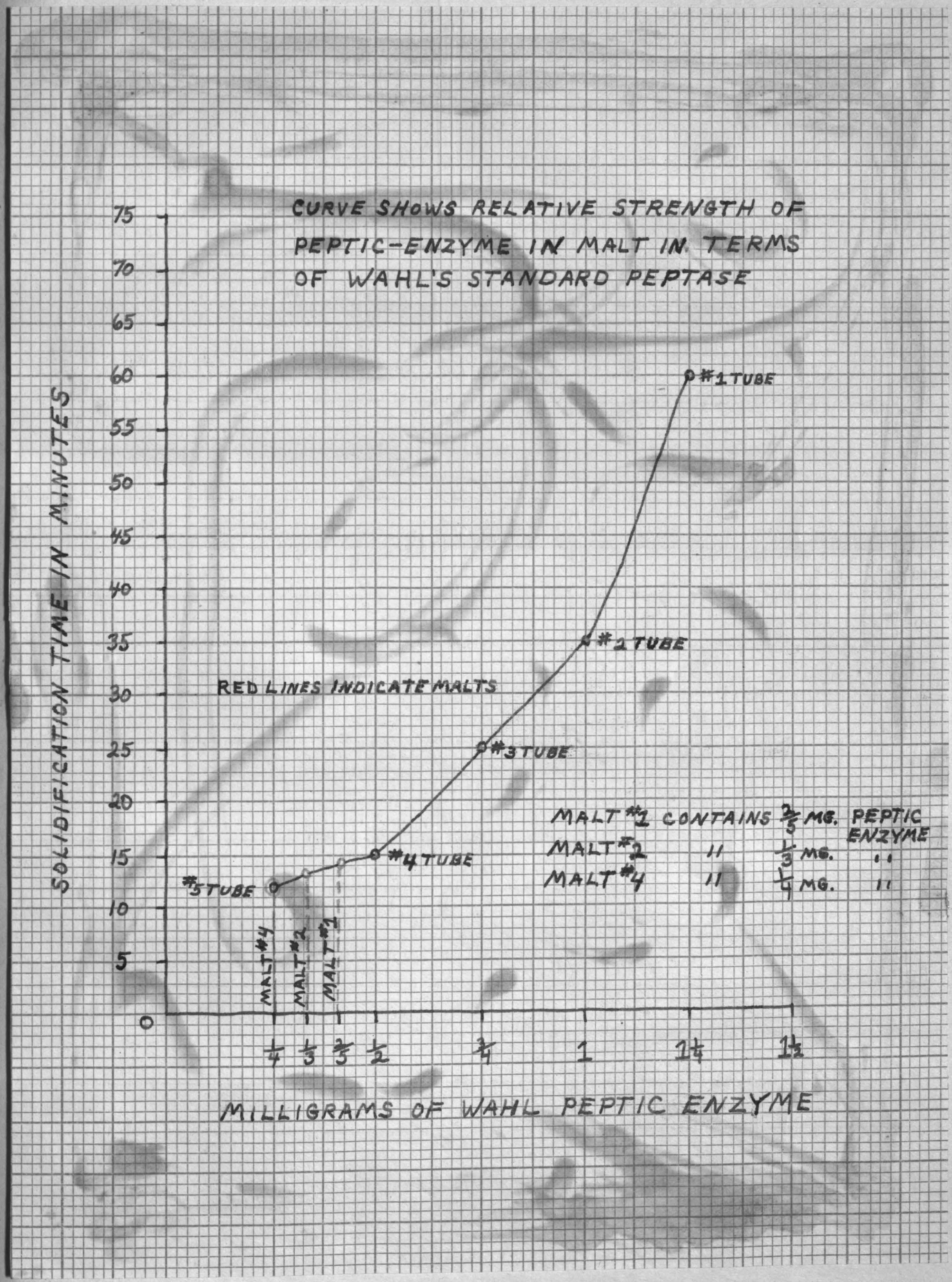
	No. of trials	Time (minutes)
MALT # X	13	Only one of the tubes solidified after being in the water bath at 21° C. This one solidified in 12 min., the other 12 tubes did not become solid after 2 hrs. in the water bath. This shows that malt #X is evidently high in peptic power, and contains more than $1\frac{1}{4}$ mg. of peptic enzyme.
MALT # 1	13	14, 14, 14, $14\frac{1}{2}$, 14, 14, 14, $13\frac{1}{2}$, 11, 14, $13\frac{1}{2}$, 14, 14. 14 minutes seems to be the correct solidification time. Comparing this time with that of the enzyme tubes--malt #1 contains about 2/5 mg. of the peptic enzyme.
MALT # 2	17	13, 13, 13, 13, 13, $12\frac{1}{2}$, $12\frac{1}{2}$, 13, $13\frac{1}{2}$, 13, 13, 13, 13, 14, 13, 13, $12\frac{1}{2}$. Assuming 13 minutes as the solidifying time--malt #2 is less in enzyme content than malt No. 1. It contains about 1/3 mg. of peptic enzyme.
MALT # 3	19	Only three tubes solidified, 18, 18, 19 min. The other tubes did not solidify after 2 hours in the water bath. Malt #3 reveals a high peptic power and is therefore similiar to malt #X.

MALT # 4

15

12, 12, 12, 12, 12, 12,
12, 12, 12, 12½, 11, 12,

Taking 12 min. as the correct time, malt # 4 contains ¼ mg. of peptic enzyme as it corresponds in time to the #5 enzyme tube.


CURVE SHOWS RELATIVE STRENGTH OF PEPTIC-ENZYME IN MALT IN TERMS OF WAHL'S STANDARD PEPTASE
SOLIDIFICATION TIME IN MINUTES
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
#1 TUBE
#2 TUBE
#3 TUBE
#4 TUBE
#5 TUBE
RED LINES INDICATE MALTS
MALT #4
MALT #2
MALT #1
MALT #1 CONTAINS 2/5 MG. PEPTIC ENZYME
MALT #2 " 1/3 MG. "
MALT #4 " 1/4 MG. "
1/4 1/3 2/5 1/2 3/4 1 1 1/4 1 1/2
MILLIGRAMS OF WAHL PEPTIC ENZYME

Experiments with Caroid.

A second type of gelatine was used together with caroid powder instead of the Wahl "isinglass" and Wahl peptase. Wahl's gelatine was found to solidify in five minutes (10 ml. of 10% gelatine plus 5 cc. of dist. water in 21° C. water bath) while the other gelatine took seven minutes to solidify. This alone shows a difference in the make-up of the two gelatines.

Thus when the caroid was added to the gelatine the solidification times of the tubes were unlike those of the Wahl gelatine and peptase. In many of the experiments with caroid the tubes did not solidify and the gelatine appeared completely modified and broken down. This would seem to indicate that the caroid is a stronger preparation than Wahl's standard peptase, for both enzymes were used in the same concentrations. In fact no solidification occurred when a lesser concentration of caroid was used.

The gelatine solutions could also be distinguished by their clarity. The Wahl gelatine was much lighter in color than the other type of gelatine.

From this one may conclude that for the proper conductance of the Wahl test--the Wahl gelatine and standard peptase should be used. Otherwise the test would have to be standardized throughout, like the Wahl method.

VI

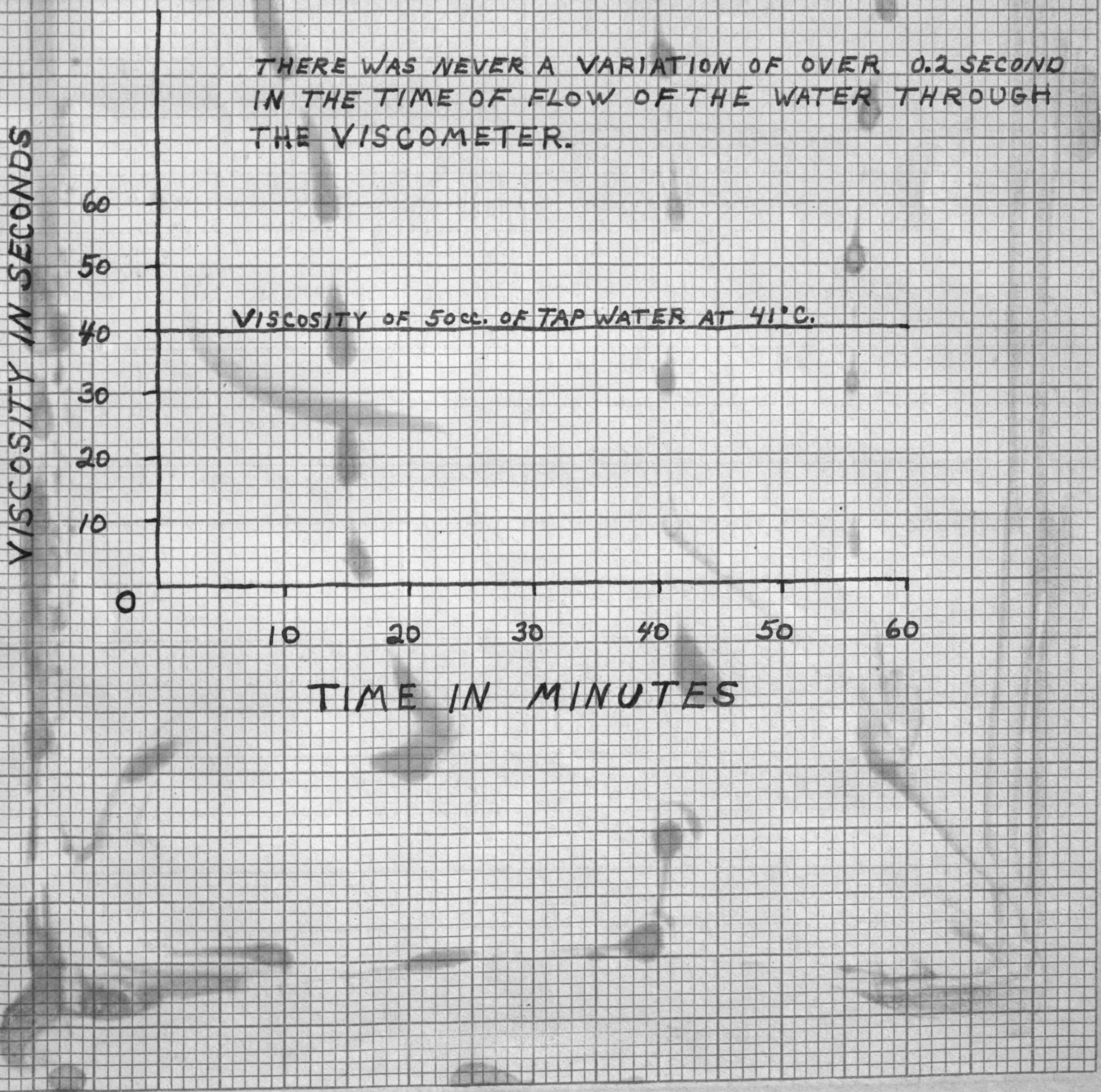
VISCOSITY DETERMINATIONS

Due to lack of time the writer was unable to make a thorough study of viscosities in regard to the modification of a substrate by pepsin.

The viscosities of tap water, a 10% gelatine solution, acid gelatine solution, and an alkaline gelatine solution were measured. The viscosity measurements were carried out with the various solutions being held at 41° C. in a water bath.

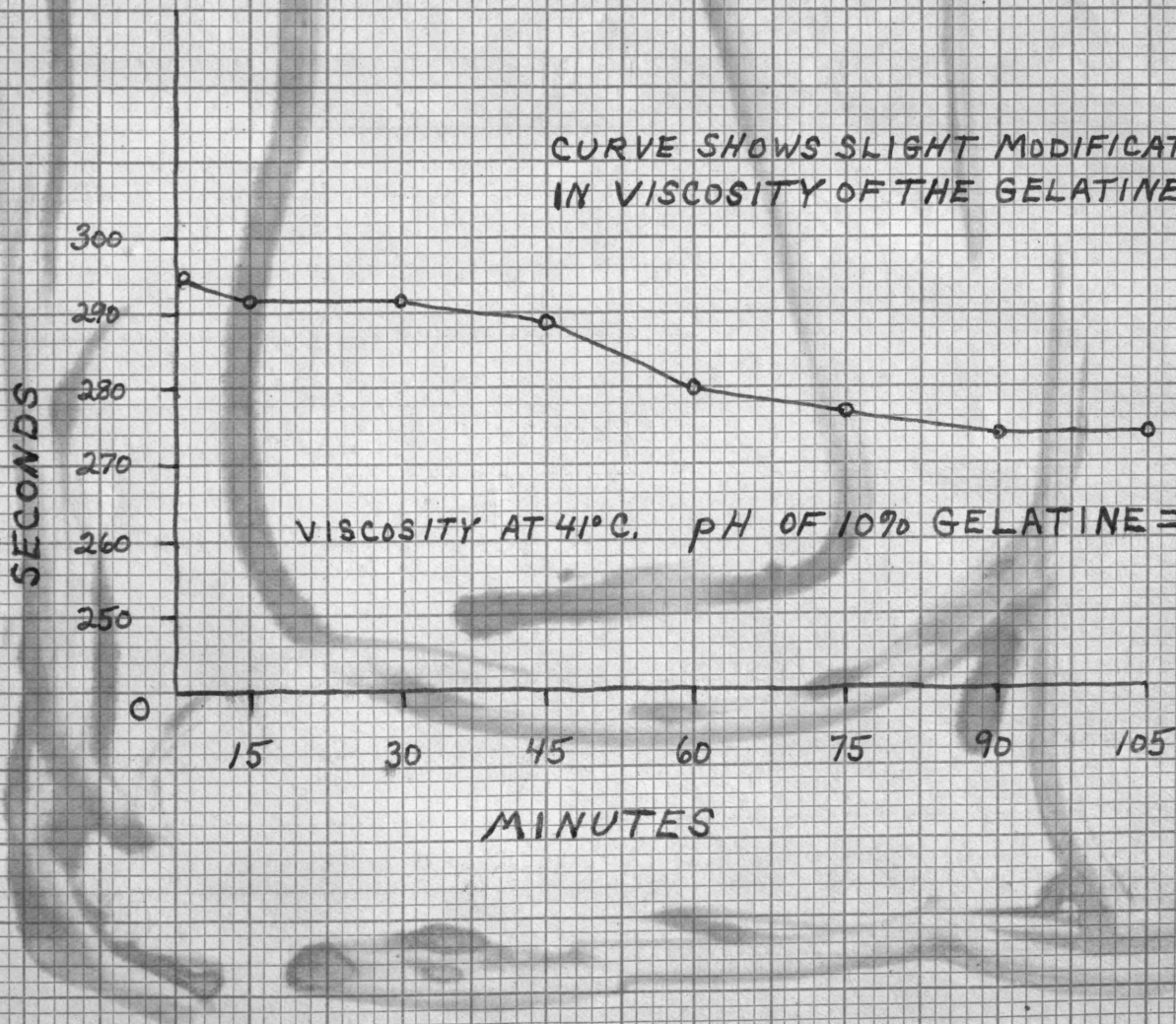
The type of viscometer used was one devised by Dr. J. R. Koch of Marquette University, Milwaukee, Wisconsin. It consisted of two Erlenmeyer flasks (150 cc.) held together by two-hole stoppers. The flasks were connected by means of a small capillary tube and an air tube. The viscosity of the solutions was measured by noting the time in seconds required for 50 cc. of solution to traverse through the capillary, from one flask to the other.

GRAPH – I


THERE WAS NEVER A VARIATION OF OVER 0.2 SECOND IN THE TIME OF FLOW OF THE WATER THROUGH THE VISCOMETER.
VISCOSITY IN SECONDS
60
50
40
30
20
10
0
VISCOSITY OF 50cc. OF TAP WATER AT 41°C.
10
20
30
40
50
60
TIME IN MINUTES

GRAPH — II

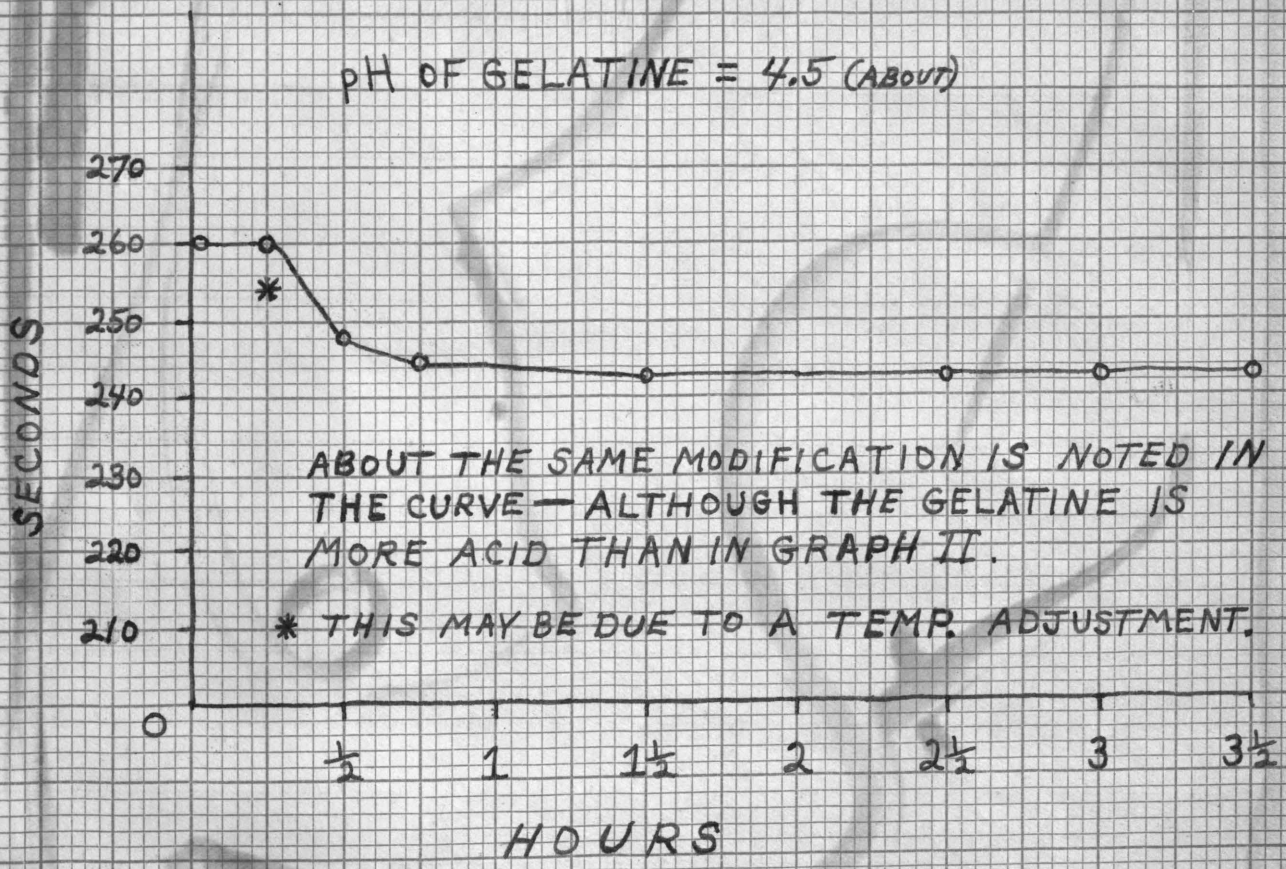
VISCOSITY CURVE FOR 10% GELATINE. - 50 cc. USED.



GRAPH — III

VISCOSITY CURVE OF 50cc. OF 10% GELATINE

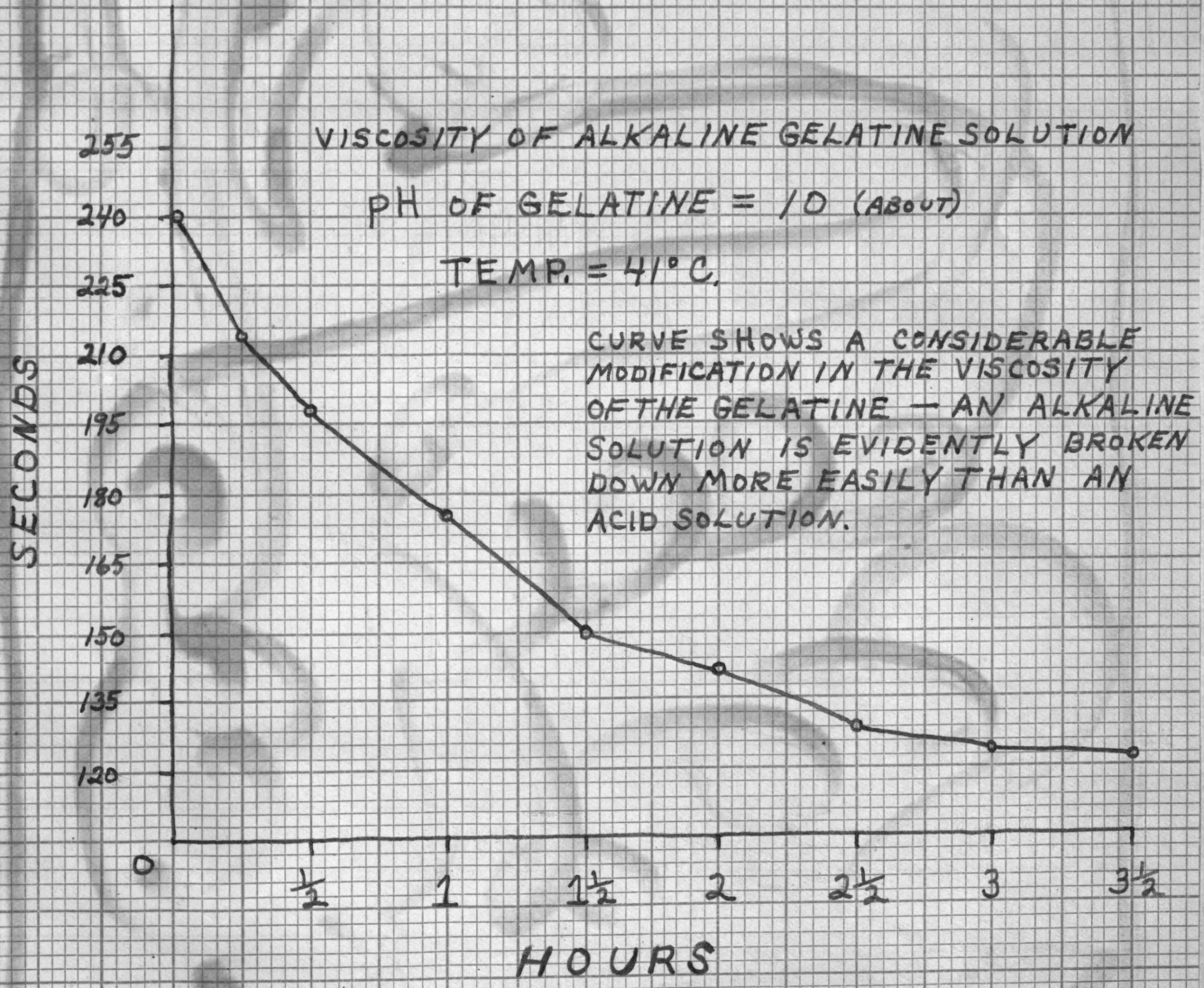
pH OF GELATINE = 4.5 (ABOUT)



ABOUT THE SAME MODIFICATION IS NOTED IN THE CURVE — ALTHOUGH THE GELATINE IS MORE ACID THAN IN GRAPH II.

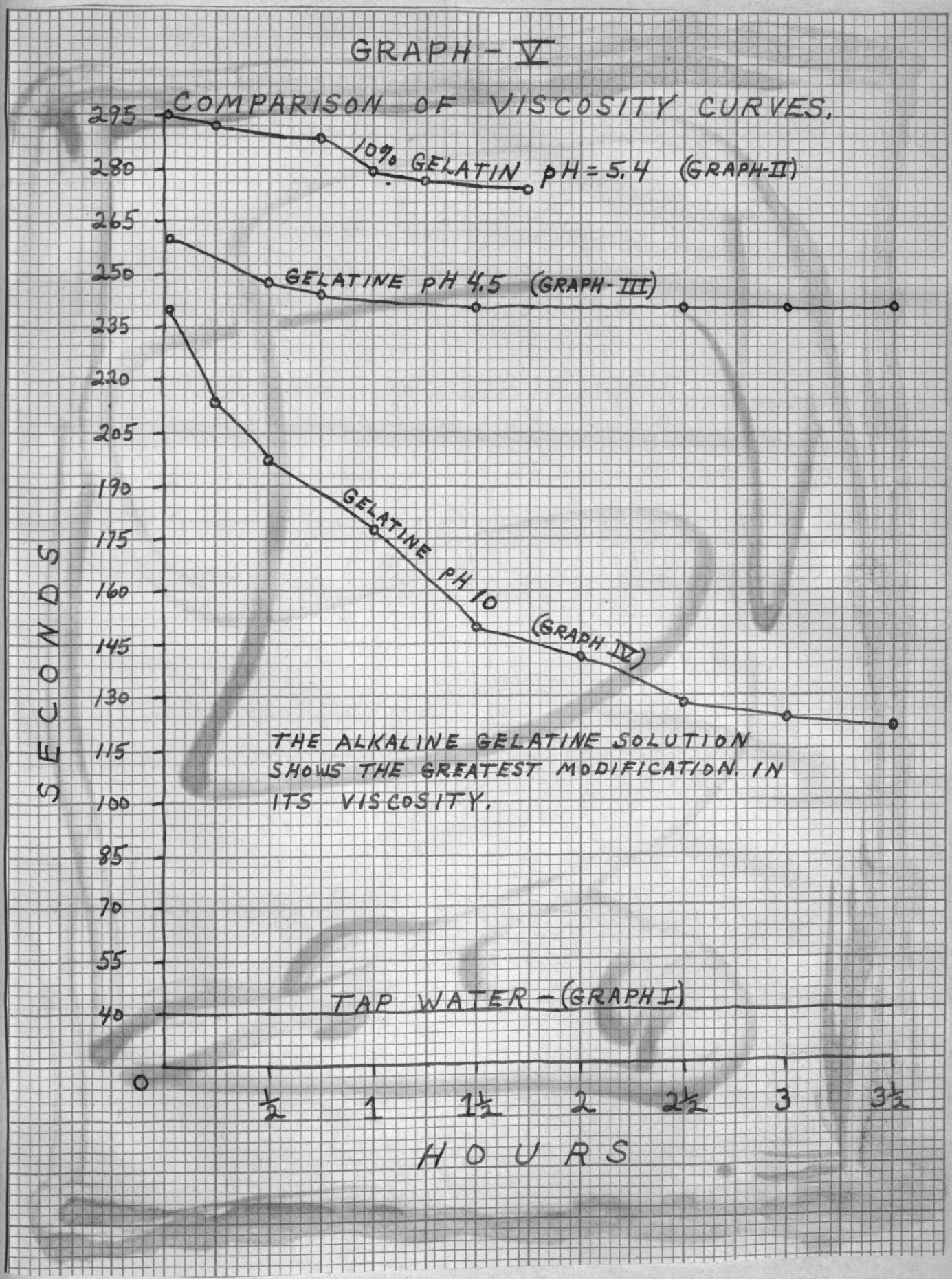
* THIS MAY BE DUE TO A TEMP. ADJUSTMENT.

GRAPH – IV


VISCOSITY OF ALKALINE GELATINE SOLUTION
pH OF GELATINE = 10 (ABOUT)
TEMP. = 41° C.
CURVE SHOWS A CONSIDERABLE MODIFICATION IN THE VISCOSITY OF THE GELATINE – AN ALKALINE SOLUTION IS EVIDENTLY BROKEN DOWN MORE EASILY THAN AN ACID SOLUTION.
SECONDS
255
240
225
210
195
180
165
150
135
120
0
½
1
1½
2
2½
3
3½
HOURS

GRAPH - V

COMPARISON OF VISCOSITY CURVES.



VII

SUMMARY

After a study of the Wahl Peptase Method and the data obtained from numerous experiments, the following appear as conclusions:

1. In order to secure uniform results the same gelatines and enzymes must be used at all times.
2. The Wahl Method, although a lengthy procedure, seems to be adaptable for routine laboratory work.
3. Malts were found to differ in their peptic powers--by means of the Wahl test.
4. A difference was noted between the Wahl "isinglass" and a second type of gelatine which was used. Both were pure preparations.
5. Wahl's standard peptase was found to be of the proper strength and more suited for the peptic tests than caroid.
6. A difference of one degree in the cooling water bath (at 21° C.) was found to alter the solidification times of the various solutions.
7. Likewise the 41° C. water bath must be kept constantly at the one temperature.
8. An increase in concentration of the enzyme brings about a further modification in the gelatine--and in a strong concentration of enzyme the gelatine is broken down to

such an extent that it will not solidify at 21° C., even though kept at this temperature for some length of time.

9. The gelatine-enzyme solutions must all be heated for 4 hours before their solidification time is determined.

10. Fresh 10% gelatine solutions must be made each time an experiment is to be carried out.

11. The significance of the bacterial lactic acid, supposedly to influence and help along the action of the enzyme, could not be substantiated by the writer.

12. A more accurate determination of peptic power seems likely, by viscosity measurements.

VIII

SUGGESTIONS FOR FURTHER RESEARCH

In studying the Wahl Peptase Method many questions have arisen which might well be a topic for research. Some suggested topics are:

1. A comparison of the action of caroid and Wahl's Peptase on Wahl's "isinglass."
2. The use of a viscosimeter to determine the peptic power in malt.
3. A study of the differences in gelatine, particularly in their solidifying strength.
4. Determination of the effect of a uniform pH value for the gelatine-enzyme solutions in the Wahl test.
5. A comparison of the enzymatic activity of papain and caroid.
6. A study to reduce the time required for the carrying out of the Wahl test.
7. To have gelatine solutions at 42° C. and put them in a viscometer at 40° C.; determine whether there is a rise in the curve as the gelatine cools to 41°, thus showing whether the curve at the beginning is due to temperature developments.

IX

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