REPORT

of

COMMITTEE ON EXAMINATION

This is to certify that we the undersigned, as a Committee of the Graduate School, have given Anant Madhar Gurjar final oral examination for the degree of Master of Science. We recommend that the degree of Master of Science be conferred upon the candidate.

Minneapolis, Minnesota

May 31 1917

R. W. Thatcher
Chairman

REPORT

of

COMMITTEE ON THESIS

The undersigned, acting as a Committee of the Graduate School, have read the accompanying thesis submitted by Anant Madhar Gurjar for the degree of Master of Science. They approve it as a thesis meeting the requirements of the Graduate School of the University of Minnesota, and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science.

Minneapolis, Minnesota
May 31. 1917

R. N. Thatcher
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STUDIES IN THE RESPIRATION OF STORED WHEAT

A THESIS

Submitted to the Faculty of the Graduate School

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by

A. M. Gurjar

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PART I.

Introduction.

In the present day methods of handling and storing large bulks of grain, the vital phenomena occasion certain difficulties that often injure its quality. Heat damage is one of the serious problems with which the grain trade is constantly confronted. Considerable effort is expended in avoiding or partially overcoming the heating of damp grain during its journey from the farm to the mill. The development of heat in such grain takes place whenever it is stored in large bulk, as in railroad cars or in elevator bins. Large quantities of heating grain enter the terminal markets every season, particularly during the summer months.

Considering the wheat kernel as a unit of life going through the general processes that are associated with vital phenomena, heat damage can be explained. The production of heat is due to the physiological oxidation of certain of the grain constituents, which process to the plant physiologist is known as 'respiration.' Respiratory activity manifests itself by its important end-products, carbon dioxide and metabolic water, and by the release of energy. Grain is a poor conductor of the heat which develops as the result of this released energy.

From such considerations it is apparent that the quantity of heat developed as a result of respiratory activity must depend upon certain specific factors of which the moisture content of the grain, and the temperature of the surrounding medium are the most important. Other factors, such as the relative consistency and condition of the grain, the influence of different gases, the

relation of oxygen to respiration, etc., possess no less significance, when we consider the chemical and physical aspects of respiration phenomena in general.

From the theoretical considerations which are discussed in the first part of this thesis, it appeared that the quantity of the respired carbon dioxide can be made an accurate measure of respiratory activity. This necessitated the employment of an exact and fairly rapid method for the determination of carbon dioxide. The description of such a method follows the theoretical discussion and forms one portion of the study.

In the experimental part, there is established the correlation between respiration and some of the more pertinent factors that are outlined above.

The Theory of Respiration.

Respiration defined:

Plant metabolism consists of assimilating and dissimilating processes; the former includes the building up of complex organic compounds out of simple inorganic ones, while the latter denotes the destruction of substances such as carbohydrates, fats and proteins, yielding carbon dioxide, water and energy as the principal end products of a process known as respiration.

Early recognition of respiration as life-phenomena.

Priestley's discovery of oxygen in 1674 was followed by Sheele's demonstration that the air exhaled by animals contained a smaller proportion of oxygen and an increased content of carbon dioxide.

The necessity of air for germination was demonstrated by Malpighi and this was followed by Sheele's proof that during the process of germination, oxygen was consumed and carbon dioxide produced as among animals.

De Saussure[1] in 1804 had shown that respiration was more active in growing plants than elsewhere and still further that it was the cause of the loss of weight to which plants are constantly subject. This important observation of De Saussure was confirmed by Boussingault's[2] results as follows:

Material	Dry Weight Before Germination	Dry Weight After Germination	Loss
46 wheat grains	1.665	0.715	0.952
10 peas	2.237	1.076	1.165

For several decades following the year 1837, respiration was understood to include photosynthesis and was designated as 'diurnal' and 'nocturnal' respiration.

Sachs[3] in 1865 advanced the following views which defined the field of respiration:

(1) That only the process concerned in the production of CO_2 could be rightly termed respiration.

(2) That respiration takes places everywhere so long as life exists.

(3) Respiration is the perpetual source from which flow the forces necessary to the internal movements.

Theories of respiration.

1. Combustion Theory.

This theory assumes that the production of heat in the animal body is due to respiration, and that of the plant was shown

by De Saussure[1] to be due to the disappearance of oxygen. Ordinary combustion also consumed oxygen and produced heat. According to this theory, respiration was taken as a gaseous exchange; "a trade between the atmosphere and the body." The theory was supported by Sachs who held that the combustion was carried on exclusively at the expense of non-nitrogenous materials. Sachs was opposed by Borodin in 1878 who suggested that the nitrogenous substances constituting protoplasm became oxidized.

After 1874, the combustion theory was weakened by the work of many investigators, including Laskowski[4], who showed that water, as well as carbon dioxide, is formed in the process of respiration and consequently that the amount of oxygen absorbed is insufficient for any complete combustion.

The ultimate dependence of the output of carbon dioxide upon the supply of oxygen seemed undeniable from the work of Broughton[5] in 1870, and of Wortman[6] in 1880; the latter having shown that seedlings when deprived of oxygen decline in their output of carbon dioxide.

In spite of the many attempts made by plant physiologists to make sharp distinctions between combustion and respiration, to the chemist, the latter process cannot signify any more than a physiological oxidation.

II. Pflüger's Cyanogen Group Theory.

Pflüger in 1875 claimed that the inspired oxygen unities in some way with the cyanogen radicles of the living substance. On the entry of oxygen, the carbon and nitrogen atoms already in an intramolecular vibration bring about a readjustment, giving

carbon dioxide and water as products of decomposition. According to this hypothesis, the protoplasm is the seat of constant changes and the instability of some of the groupings of its constituent atoms causes decomposition to be of an explosive character. The entry of oxygen increases this instability and provokes the explosive decomposition.

III. Verworn's Biogen Theory.

This is a modification of Pflüger's theory and was extended by Verworn[8] in 1894. Verworn associated the entire action with the so-called 'biogen' molecules. The oxygen entering into the biogen molecule gives it its maximum power of decomposition so that only slight impulses are required to bring about the union of the atoms of oxygen with the carbon in the cyanogen.

IV. Enzyme Theory of Respiration.

A. Fermentation and respiration compared.

The decomposition of sugars and the consequent formation of alcohol and carbon dioxide has been for centuries known as fermentation. Since the period of 1830-40, the process of fermentation was recognized to be due to the presence of living cells. Büchner's discovery of zymase in 1896 assigned to fermentation a new role in plant metabolism, since this zymase was capable of transforming glucose into alcohol and carbon dioxide. Yeast and diastase or 'organised' and 'un-organised' were the two varieties of active agents concerned in fermentation. For the latter class Kühne in 1878 suggested the name "Enzyme."

Pasteur in 1861 demonstrated that yeast and several species of bacteria are able to live in the absence of oxygen

and that such life is associated with the carrying on of the process of fermentation.

Pfeffer[9] in 1878 proposed the name 'intra molecular respiration' for that respiration occurring in the absence of oxygen, because the energy and carbon dioxide come from the destruction of the molecule from which carbon dioxide arises. He held the view that aerabic and intra molecular respiration were genetically connected and that the existence of the intra molecular respiration was the reason for the aerabic.

Among the many investigators who confirmed and supplemented Pasteur's work, the findings of Lechartier[1] in 1869, Bellamy[1] in 1872, Brefeld[1] in 1876, De Luca[1] in 1878, added much to the general theory, as these authors worked with various succulent fruits, potatoes, grains of wheat, and different parts of plants such as seeds, leaves, and branches. Many products which do not occur in the normal respiration were found to accompany the intra-molecular respiration. Besides alcohol, De Luca found hydrogen and marsh gas while Boehm in 1875 observed the occurrence of ammonia.

Nabokich[10] (1903) held that there are two kinds of intra-molecular respiration; that one is a true alcoholic fermentation of glucose; and that the other is a fermentation of glucose with the additional production of organic acids such as lactic acids resulting in a large excess of carbon dioxide over alcohol, thus pointing to the opinion that fertmentation is only a part of anaerobic respiration.

The work of Kostytchew[11] has furnished the final proof that respiration in higher plants occurs in two stages; a primary -

anaerabic stage, and a secondary stage, which results in the oxidation of the products of the first stage.

Kostytchew[11] has further shown that anaerabic respiration is not always identical with alcoholic fermentation; yet the latter process plays the most important part in the anerabic respiration. Since zymase is an established enzyme of alcoholic fermentation, it may be classed as a respiratory enzyme.

Palladin and others have pointed out that the entire process of respiration is due to the summation of enzyme activities.

B. Enzymes and Respiration.

Schoenbein's discovery of ozone in 1840 gave a real basis for a better understanding of the guaicum reaction and oxygen activation in plants.

Traub in 1877 first introduced the term 'oxidizing ferment' which combined with free oxygen, formed unstable compounds which in turn gave up their oxygen to other less readily oxidizable substances. Bertrand expressed the specific nature of the oxidizing ferments by using the term "oxidases." For still other ferments which rendered active the oxygen of hydrogen perioxide, Linossier in 1898 gave the name "peroxidases."

According to Engler[12], the phenomena of oxygen activation is due to the oxidation of the second substance - the acceptor - by the peroxide (perioxide) resulting from the antoxidation of the carrier. Thus when an antoxidizable substance, A, finds itself in contact with oxygen and a second oxidizable substance B, the following changes would occur

$$A + O_2 = AO_2$$

$$\text{and } AO_2 + B = AO - BO$$

In this way a substance incapable of combining directly with oxygen may be oxidized through the intervention of another substance.

According to Bach and Chodat[13], peroxidases can not only activate oxygen of hydrogen peroxide but also that of organic peroxides, thus indicating that oxidases are not single enzymes but mixtures of peroxidases and peroxide forming substances designated by a group name "oxygenases."

Regarding the activation of hydrogen peroxide, Kastle[14] (in his exhaustive monograph on Oxidases) states his conclusion that it is mainly due to its tendency to unite directly with oxidizable substances "forming thereby either a peroxide or some other complex which tends to part with its oxygen more easily than the hydrogen peroxide itself.

Among the oxidizing enzymes we have still another class - namely, the catalases. The term was introduced by Loew who proved that the decomposition of hydrogen peroxide by plant and animal tissues was due to this enzyme.

Mechanism of Respiration and Enzyme Action.

The Chromogen Theory of Palladin.

The mechanism of respiration has been recently explained by Palladin through what may be termed the 'Chromogen Theory."

Palladin[15] and his co-workers have held that oxidases work upon colorless sap soluble chromogens. They conceived of this process as taking in of oxygen by a readily oxidizable

substance to form a peroxide which is then split up by oxidases, yielding its oxygen for the oxidation of reducing substance, elaborated by the protoplasm.

Palladin's chromogen theory came from the peculiar difficulty that though the mechanism of respiration was mostly explained by assigning the oxidative forces a role in cell oxidation, the latter were capable of accelerating the oxidation only of aromatic compounds which are entirely foreign to plant tissues or present only in insignificant amounts; which fact further meant that the oxidative forces exerted no action on the ordinary substances like sugar, consumed in respiration.

Palladin's[15] hypothesis met this difficulty as follows:- The first product of sugar fermentation is some aldehyde. This can be partially converted into acid by the Cannizzaro-reaction or totally into acid by the Schardinger mechanism. By means of carboxylase, carbon dioxide is split off from the carboxyl in the acids formed. Thus, the total carbon dioxide expired during respiration is anaerobic in origin. The Schardinger mechanism requires the presence of an acceptor for the hydrogen formed by the water decomposition. In Palladin's hypothesis, this function is assigned to the respiratory pigements which are reduced to chromogens. At this point, oxidases enter the field of action and oxidize the chromogen back to pigment by the removal of hydrogen and the formation of water. All of the oxygen consumed during respiration is used for the oxidation of the hydrogen in the pigment acceptors. The water arising during respiration is entirely aerobic in origin contrast with the anerobic carbon dioxide. Since the chromogens are aromatic substances, they fall

into the same general class of substances acted upon by the demonstrable oxidases (Cited by Appleman, Md.Bull.).

Palladin[15] has illustrated the anaerabic and aerobic stages of the above hypothesis by the following equations.

I. Anaerobic stage

$C_6H_{12}O_6 + 6H_2O + 12R$ (Resp.Pigments) $= 6CO_2 + 12RH_2$(Chromogens).

II. Aerobic stage.

$12RH_2 + 6O = 12H_2O + 12R$

Catalases and Respiration.

The Chlorolphyll Function.

According to the researches of Usher and Priestley, there are three agencies concerned in the chlorophyll action, namely - protoplasm of the chloroplast, the chlorophyll itself and a catalase. By means of the pigment acting as both optical and chemical sensitiser, light energy is employed to cause reaction between CO_2 and water in such a manner that formaldehyde and hydrogen peroxide are formed. Both of these bodies are toxic and if allowed to accumulate, the reaction soon comes to an end. The formaldehyde is polymerized by the protoplasm of the chloroplast, however, and the hydrogen peroxide is split into oxygen and water by the catalase.

Loew, in considering the significance of catalase, pointed out that hydrogen peroxide results as either a primary or secondary product in the antoxidation of many readily oxidizable substances. The activity of the catalase in the physiological oxidation was established by Lesser and Zieger who worked with organs and tissues of animals.

Woker and Begemann[16] have advanced a new conception regarding the protective action of the catalase. These authors think that catalase and peroxidase-actions are only different manifestations of oxygenase. Accordingly, they conceive the peroxidase, catalase and phenolase as of aldehyde nature.

This view of Woker, and Begemann has been disputed by Bach who claims that each of these enzymes is an individual whose reactions cannot be regarded as different manifestations of an aldehyde.

Appleman[17] investigated the relation of oxidases and catalases to respiration. He worked with potato tubers subject to different conditions of temperature, etc. He found that the catalase activity in the potato juice showed a very striking correlation with respiratory activity in the tubers.

Chemistry of Respiration.

Hoppe Seyler[18] in 1876 suggested an hypothesis that in all organisms, respiration is primarily anaerobic. This same view was advanced by Detmer in 1879, who held that all vital processes consist in a break-down of lobile compounds, the decomposition of which subsequently undergoes respiration.

It goes therefore, without saying, that during respiration certain very complex chemical changes take place. Exactly what these changes are, has been a matter of much research and speculation. The protoplasts of individual cells gradually become disorganized during respiration and according to C. de Candolle[2] (1895) life is simply subdued during the process.

But the period of this reduced activity is comparatively short for respiration soon ceases and life becomes wholly latent. We may notice the contrast between the oxidation and hydrolytic processes; the former in the presence and the latter in the absence of oxygen for supplying the energy. In an article on "Life Without Oxygen," C. D. Snyder[18] states that the fundamental chemical processes of the cell in all organisms are anerobic and the phenomena of oxidation are secondary and have been built up in the course of evolution. Need of oxygen appears to increase with increasing complexity of chemical and morphological organization.

Respiratory Ratio.

From the simple observation that starch and sugar disappear during respiration, it was at one time concluded that if these compounds would be completely burnt, we must expect carbon dioxide and water as final products according to the equation $C_6H_{10}O_5 + 6O_2 = 6CO_2 - 5H_2O$, thus making the gaseous exchange a unity. De Saussure[1] in 1804 had suggested that the loss in the weight of germinating seeds was due to the loss of water during respiration. This view was confirmed by Laskrosky in 1874. Rübner in 1885 demonstrated that the respiratory ratio varied at different temperatures. Purjnwicz[37] (1900) having found wide variations in the intake of oxygen and the output of carbon dioxide, expressed his conviction that the respiratory ratio had no value for indicating the actual course of respiration. He therefore separated the taking up of oxygen and production of carbon dioxide as two processes. After

Purjnwicz's work it was established that in the physiological combustion, there were many intermediate actions between the absorption of oxygen and the excretion of carbon dioxide. Besides carbon dioxide and water, there must be other bodies as well, such as organic acids.

Role of Carbohydrates.

With regard to the actual fate of materials, carbohydrates are not oxidized in the ordinary sense, i.e., oxygen of the air does not combine directly with carbon or with carbon monoxide to form carbon dioxide or with hydrogen to form water. E. F. Armstrong[19] has shown that substances do not undergo direct oxidation but hydroxilation, i.e., with consequent splitting into various intermediate products such as carbon monoxide, hydrogen peroxide, carbonic acid and water. The carbohydrates are thus decomposed by continued hydroxylation, and diastase in some way fecilitates the dissociation. The socalled zymase is the last one to act upon glucose, forming lactic acid which still further breaks into alcohol and carbon dioxide. For the more specific enzyme action it is assumed that water combines with starch under the action of diastase. The hydrolysis of glucose by zymose has been designated by the term 'fermentation' which is merely an incomplete oxidation while respiration is the complete combustion of these organic materials.

According to Harden and Young[20], the hydrolysis of carbohydrates is not so simple as it appears. These authors claim that phosphoric acid is essential to the normal zymase activity. The hexoses interact with the phosphates (especially

in the case of yeast ferment) forming hexose phosphate simultaneously with CO and alcohol. The following reaction illustrates this point.

I. $2C_6H_{12}O_6 + 2R_2HPO_4 = 2CO_2 + 2C_2H_5OH + C_6H_{10}O_4(PO_4R)_2 + H_2O$

The hexosephosphate is then hydrolysed by hexophosphatase.

II. $C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = C_6H_{12}O_6 + 2R_2HPO_4$

Harden and Young also claim that zymase is quite unable ferment sugar unless a co-enzyme is present. This co-enzyme is a thermostable substance and can be removed from yeast juice by dialysis.

Büchner and Meisenheimer[38] believe that di-hydroxy-acetone

$$\begin{array}{l} CH_2OH \\ | \\ CO \\ | \\ CH_2OH \end{array}$$

and not the lactic acid is the chief intermediary. Still another product appears to be glycerice aldehyde

$$\begin{array}{l} CH_2OH \\ | \\ CHOH \\ | \\ CHO \end{array}$$

which may be converted into methyl glyoxal (CH_3-Co-CHO). This in turn splits up into pyruvic acid and pyruvic alcohol. The former splits into carbon dioxide and acetaldehyde which is then reduced to final product ethyl alcohol. Traces of lactic acid are formed as a reaction perhaps by the hydration. R. H. A. Plimmer[21] illustrates this transformation as follows:

$$\begin{array}{l} CH_3 \\ | \\ CHOH \\ | \\ COOH \end{array} \qquad \begin{array}{l} CH_3 \\ | \\ CO \\ | \\ COOH \end{array} \qquad \begin{array}{l} CH_3 \\ | \\ CHO \\ | \\ CO_2 \end{array} \qquad \begin{array}{l} CH_3 \\ | \\ CH_2OH \\ | \end{array}$$

As to the quantities of CO_2 and alcohol produced during respiration, the work of Godlewski and Polzeniusz (1909) has furnished considerable evidence. In the absence of oxygen carbon dioxide and alcohol are formed in nearly the same proportion as in alcoholic fermentation. This proportion is expressed in the following reaction

$$C_6H_{12}O_6 = 2CO_2 + 2C_2H_5OH.$$

Role of Carbohydrates in Anaerobic Respiration and Fermentation.

The d-ethylidine lactic acid or sarco-lactic acid is a product of both alcoholic fermentation and protein respiration. Such an analogy had already been suggested by Pasteur.

Maize[39] and Stoklasa[40] held controversial views on the inter-relationship between anaerobic respiration and fermentation; the former holding the process of fermentation to be a neutritive one, sugar being assimilable when fermented and the nassent alcohol thus being made available; while Stoklasa considered fermentation to be merely anaerobic respiration and essentially a process for the immediate release of energy.

Godlewski[41] (1911) concluded that anaerobic respiration is identical with alcoholic fermentation or at least dependent on it.

The process of oxidative decomposition of food substances is therefore separable into two stages. The first stage consists of the anaerobic production of alcohol and carbon dioxide while the second stage comprises the aerobic oxidation of alcohol produced in the first stage; and this is where the chromogen theory

of Palladin already quoted, comes in. The respiratory pigments held by Palladin as an agency in the oxidation of alcohol are present originally as glucosides and liberated by hydrolysis. The respiratory chromogens according to Palladin, are cyclic compounds bound to carbohydrates in the form of insoluble glucosides. Glucoside splitting enzymes separate the cyclic compounds which by the aid of the oxydases are then enabled to take up oxygen from the air to give it up again later under the influence of reducing substances.

Proteins and Respiration.

As to the second important group of food substances, namely, proteins, there is very little direct evidence as to its utilization in respiration. Construction of protein, i.e., by substituting H atoms by NH_2 groups is quite analogous to that of carbohydrates. In the cleavage process also, we have putrefaction as parallel to fermentation; each brought about by a separate group of enzymes.

The only direct experimental evidence on the point in question is that furnished by Wehmer[22] (1892) who fed the fungus Aspergillus on peptone as a source for nitrogen and sugar for carbon. The fungus also supplied all its wants as to carbon and nitrogen when peptone alone was given to it. Under these circumstances, functions carried by sugar must have been undertaken by peptone and it appeared that nitrogen of the peptone was transformed to ammonia and excreted.

In his extensive work on respiration of fruits, H. E. Gore[23] came to conclusions quite opposite to the evidence just cited.

He found that during the ripening of bananas the conspicuous change is that of starch into sugars. During this period the respiration rate increases manyfold, becoming greatest when the rate of starch hydrolysis is most rapid. The starch hydrolysis gradually slackens, later ceasing altogether. The respiration also becomes slower but still remains far more active than in the green fruit. Next to starch and respiration changes, most distinct are those of water. Absorption of water by the pulp amounted to 0.512 to 0.782 per cent as a net result of respiration and starch hydrolysis. The osmotic pressure between the peel and pulp as the sugar is converted becomes very great. The rate of evolution of carbon dioxide from green to post ripening varied from 0.146 to 0.091 per kg. per hour.

Gore[23] further concluded that the quantity of ash, portein, and other extract underwent but slight changes during the ripening. Pentosans decreased markedly in the pulp but remained little changed in the peel.

Still, very recently, Langworthy and Milner[24] in studying the ripening bananas with the respiratory calorimeter came to an identical conclusion. These anthers found the respiratory quotient exactly equivalent to that involved in the combustion of carbyhydrates. From this they concluded that other constituents of the banana as tannin compounds, aromatic and flavoring bodies and proteins were not concerned in the energy transformation to any extent.

Significance of Respiration.

Production of energy.

The result of respiration is the release of energy for plant metabolism. To designate this effect, Barnes[25] has used the expression "Aerobic, anaerobic and fermentative energesis."

Since the physiological activities are carried on at the expense of energy this must be made available in plant tissues. It is through the respiration that the Sun's energy stored up in the complex compounds by photosynthesis is released in those tissues where it is needed. Starches, fats and proteins are the products of assimilation but by and for themselves there are merely innert. For these to be set in motion and for a new structure to actually come into existence, energy is necessary and it is respiration which releases this in the organism. Specifically, the loss of substance which results in addition from respiration serves to develop forces by means of which the atoms and molecules of the remaining substance are set in those movements from which growth takes place.

Production of Metabolic Water.

Respiration plays an equally important part in the production of metabolic water. By the latter term is meant water produced by the complete oxidation of carbohydrates, fats and proteins. Complete oxidation of these substances actually produces water equal to nine times the weight of their hydrogen. For instance: one hundred parts of cellulos or starch $(C_6H_{10}O_5)_n$ containing 6.17 per cent hydrogen gives 55.5 parts of water;

one hundred parts of dextrose containing 6.66% hydrogen gives 60 parts of water. Proteins when completely oxidised yield from 60 to 65% of water. Since respiration is a function peculiar to protoplasm and since the latter consists of distinct cellular units, the production of water is confined to the interior of such cells.

The immediate effect of respiration upon metabolic water, as is shown by Babcock[26], is to remove by oxidation and dehydration a portion of the nutrient dissolved in the cell fluid, replacing them in part by water and thus to reduce the concentration of the solution within the cell wall below that of the surrounding fluid. In consequence of this, there is established an osmotic movement toward an active cell - and of water in the opposite direction. So long as suitable nutrients are supplied and respiration is continued, this condition is maintained. It is this partial replacement of nutrients by metabolic water that determines the direction of the movement and insures a constant supply to all respiring cells.

Babcock[26] further points out that the specific enzymes required for the conversion of stored nutrients of a seed into available forms are absent in the immature seed and only appear in the mature seed after direct respiration is established, the rate being quite proportional to the maturity.

In the regular food cycle of plants from the insoluble carbohydrates formed originally in the leaves by photosynthesis to the soluble dextrose, osmosis is continually going on in all parts of the plant. Oxidation of soluble sugars and dehydration gives rise to cellulose, starch sucrose, etc. In all these

processes which may occur indefinitely, water is transferred in organic combination from the leaves to every growing cell, and it is respiration that results in the production of this water within the cell walls.

PART II.

The Adaptation of Truog's Method for the Determination of Carbon Dioxide to Plant Respiration Studies.

As pointed out in the theoretical discussion, carbon dioxide can be regarded as an accurate, and at the same time the most readily determinable, index of respiratory activity available in laboratories not provided with an elaborate respiration calorimeter. Satisfactory methods for the determination of carbon dioxide are accordingly of interest to plant physiologists and biochemists who are concerned with this phase of phtyochemistry.

In the preliminary work on the study of respiration of stored grain, it became evident that there were two requisites which the method for the dertmination of carbon dioxide must satisfy in order to be applicable to this purpose. First, since the plant tissues are constantly respiring, in determining the rate of respiration it is necessary that the accumulated carbon dioxide be rapidly removed from the respiration chamber. Second, the method must accomodate thw wide variations in the quantity of carbon dioxide to be determined, without materially sacrificing accuracy.

The customary, and most convenient method of determining the quantity of carbon dioxide in the atmosphere of the respiration chamber, is to sweep CO_2-free air through it, and absorb the respired CO_2 in some form of absorption train. The conventional absorption train, in which small potash bulbs are employed,

is of limited value because of the slow rate at which the gases must be passed through it. It cannot be employed where the time element is significant. The method of Truog[27] in which the carbon dioxide is absorbed in a measured quantity of N/4 $Ba(OH)_2$ solution contained in a special absorption tower, and the residual barium hydroxide titrated against a standard HCl solution, seemed best adapted to this purpose. The advantages of this method, together with a discussion of titrimetric methods, are presented by Truog. In assembling the apparatus certain difficulties were encountered, however, and an attempt was made to overcome them. Thus the original form of absorption tower did not provide an adequate means for transferring the standard $Ba(OH)_2$ solution, and CO_2-free water from the stock bottles to the absorption tower without exposing them to the atmosphere. An automatic pipette was accordingly constructed, which was connected to both the stock bottle and the tower in such a manner that the $Ba(OH)_2$ solution could be transferred and measured through a closed system protected from the laboratory air by soda-lime tubes. A convenient arrangement for rendering the wash-water free from carbon dioxide without disconnecting the reservoir was also assembled. These additions rendered the apparatus more efficient for plant respiration studies, and they are described in detail in this paper.

Description of the Apparatus.

The complete apparatus is illustrated in figure No. 1. The absorption flask and tower tube are similar to those employed by Truog, except that the tube is provided with an adapter (D) at

the top. This adapter has a belled top, which not only affords a tight connection with the rubber stopper (E), but can be readily separated from the stopper in disconnecting the several parts of the apparatus. This adapter is connected to the tube (C) by means of a packing of pure gum tubing. The stopper (E) has two holes, one of which admits the tip of the automatic pipette (H), and the other the lower tube of the U-tube (F). The automatic pipette is connected through a three-way cock to the stock bottle containing the $Ba(OH)_2$ solution, and is filled by raising the pressure in the bottle by means of the rubber bulb (L), opening the pinch-cock (2), and turning the glass cock to the proper position. The pipette is drained by turning the stop-cock over, and a mouth piece (J) is provided with an intermediate soda-lime tube (S).

One of the arms of the U-tube (F) is fused to a cylindrical separatory funnel (G), which serves as a container for the water used in diluting the $Ba(OH)_2$ solution and in washing out the tower when the aspiration is completed. This funnel is filled by raising the pressure in the large stock bottle of water with the rubber bulb (L), and opening the pinch-cock (3). The other arm of the U-tube is connected to the intake of the meter (M), which in turn is connected to the aspirator. This arrangement makes unnecessary a third hole in the stopper (E). The U-tube and automatic pipette are supported by clamps attached to a standard.

Technique of Determination.

Clean glass beads are placed in the tower (C) to a depth of from 16 to 20 inches, the depth depending upon the

quantity of $Ba(OH)_2$ solution to be used. The stopper (E) is placed tightly in the adapter (D) at the toper of the tower, and carbon dioxide-free air is aspirated rapidly through the beads and tower to free them of carbon dioxide. After about 5 minutes of vigorous aspiration the suction is released, and the automatic pipette is filled to the mark with $Ba(OH)_2$ solution. Should the solution overrun the mark on the pipette stem, on releasing the air pressure by opening pinch-cock (1) the solution will run back in the bottle by opening pinch-cock (2) and the proper level may be reached in the pipette. The three-way cock of the automatic pipette is then turned over, and the $Ba(OH)_2$ solution allowed to flow into the tower. The last drop is removed by blowing twice through the mouth-piece (J). It was found that when blown uniformly, the pipette would deliver with a maximum variation of 0.01 c.c. Since one cubic centimeter of N/4 $Ba(OH)_2$ is equivalent to about 6 milligrams of CO_2, 25 c.c. will absorb about 125 milligrams of CO_2 and leave a safe excess of $Ba(OH)_2$. Should the quantity of CO_2 to be absorbed exceed this amount, two charges from the pipette must be transferred to the tower; if less, one charge is employed, and about an equal volume of CO_2-free water. The tubulure of the suction flash is then connected to the respiration chamber, a minimum of rubber tubing being exposed to the current of gases. In the bulk-grain respiration studies, an 18" calcium chloride tower (A) has been employwed as a respiration chamber. The latter is connected at the top to a gas washing device charged with 50% KOH solution for removing the CO_2 in the air employed for flushing out the air in the chamber. Pinch-cock (6) being opened, aspiration is begun at a slow rate.

the gases being drawn through the meter (M). The meter readings show the volume of air which has been drawn through the system. With bulk grain ten volumes of the capacity of the respiratory chamber completely flushes out the carbon dioxide in the latter. After about one volume of air has been drawn through the tower the rate of aspiration may be increased. It has required about 45 mintues to complete the aspiration in the studies mentioned.

The tower is then disconnected at the stopper (E) and elevated from the suction flash sufficiently to permit the beads and $Ba(OH)_2$ solution to flow down into the flask. About 50 cc. of carbon dioxide-free water are run from the separatory funnel (G) down the sides of the tower, while the latter is being revolved in such a manner as to insure thorough washing of its inner wall. The suction flask is disconnected from the respiration chamber, the tower lifted out of the flask, and the residual $Ba(OH)_2$ titrated against a standard HCl solution, using phenolphthalein as an indicator. Checks of the apparatus and method, using C. P. $CaCO_3$ have demonstrated their desirability and accuracy for this purpose.

Preparing the Carbon Dioxide-Free Water.

The stock bottle for the carbon dioxide-free water may be easily filled without removing the stopper by disconnecting the rubber tube to which pinch-cock (3) is attached, and connecting the tube to which it is attached to the aspirator. When suction is applied by means of the latter, water is rapidly drawn into the bottle. The water may be rendered free from

carbon dioxide by closing pinch-cock (3), and opening pinch-cock (5). Ait may thus be passed vigorously through the water, about 30 minutes of aspiration rendering it sufficiently pure for this purpose. To determine its freedom from carbon dioxide, 100 c.c. are titrated against N/20 KOH, using phenolphthalein as an indicator. It should not require more than 0.2 c.c. of the KOH solution to produce a permanent pink coloration.

The apparatus arranged and operated in the manner described will satisfy the requirements outlined in the second paragraph. It has been employed in determining carbon dioxide accumulated by the incubated wheat.

PART III.

EXPERIMENTAL.

Technique of dampening and incubating the grain for the accumulation of carbon dioxide.

The samples were first brought to approximately the desired moisture content by adding water, the quantity of water being calculated on the basis of the moisture content of the original grain. After thoroughly mixing the calculated quantity of water, the grain was allowed to stand in sealed jars for 72 hours in order to secure uniform distribution of the water through the kernels. The 72 hour period was decided upon from preliminary work on changes in specific gravity after the addition of water. It was found that swelling of the kernel reached the maximum in 24 hours and the specific gravity and kernel volume remained constant after that period. This would indicate that the added water was evenly distributed through the kernel at the end of 24 hours.

At the end of the 72 hour period the grain samples were placed in calcium chloride towers such as shown in Figure 1 and incubated in thermostats. All rubber surfaces exposed to carbon dioxide were first painted with paraffin, as were the stopper joints. The charges of wheat consisted, in case of low moisture limits, of from 500 to 700 grams, and for the high moisture limits, of from 250 to 300 grams, the intent being to secure accurately determinable quantities of carbon dioxide. After sealing in the stoppers, the atmospheric carbon dioxide within the tower was swept out by carbon-dioxide

free air. In this condition the outlet tubes of the tower were sealed, and the towers incubated. At the time of incubation, samples of grain were also taken for the moisture determination upon which the final calculations were based.

The period of incubation was fixed at four days, the exact number of hours being noted at the time of removing the respiration chambers from the thermostat. The final calculations are based upon the quantity of CO_2 respired per 100 grams of dry matter in each 24 hours. The total quantity of carbon dioxide respired by the grain was determined at the end of the incubation period by connecting the respiration chamber to the absorption tower, as described above.

The relation of the moisture content of wheat to the rate of respiration.

It is apparent from the theoretical consideration that the moisture content of plant tissues bears an important relation to the process of physiological oxidation. Plant physiologists in the past, in measuring the respiratory activity of seeds, have dealt mainly with germinating seeds, water being absorbed to practically the maximum capacity at this stage. Extensive work by various authors, dealing with fruits and seedings, as already pointed out, involved very high limits of water, which, though apparently playing an important part, has not been investigated as a controlling factor.

Barnes[25], in his theory of respiration, states that the function of water is to effect the addition of hydroxyl groups from the water, shifting the atomic groups within the molecule;

the dissociation of the protoplasm follows, and energy is liberated for growth and movement. Catalytic agents like enzymes are the usual accelerators.

Babcock[26] found that the hydrolytic changes consist in adding the elements of water the to molecular structure of the nutrient. Further, these changes are affected by the action of specific enzymes peculiar to each type of organism which is produced by living cells.

The form in which water exists in the kernels is of significance in this connection. Organic colloids have the property of imbibing considerable quantities of water. Naegeli[28] advanced an hypothesis to account for the phenomena of imbibition. He maintains that all organized tissues of plants that are capable of imbibition consist of minute molecular aggregation designed as micellae, between which water enters, forcing them apart, and thus increasing the volume of tissues when they are immersed in water. The entrance of water between the micellae, between which water enters, forcing them apart, and thus increasing the volume of tissues when they are immersed in water. The entrance of water between the micellae is supposed to be effected by some other force than capillarity, since it does not take place when the tissues are immersed in such liquids as absolute alcohol or anhydrous glycerin.

Babcock[26] points out the inadequany of the Naegeli hypothesis in the fact that plant tissue does not swell when immersed in absolute alcohol or anhydrous glycerine both of which adhere to the clean, dry surface of bodies composed of such tissues. He accounts for the phenomena of imbibation by a

direct molecular combination of the substances composing the tissues of organized bodies and water.

Bailey[29] discussed the physico-chemical considerations involved in the relation of the moisture content of wheat to the rate of respiration. Attention is called to the fact that organic colloids of the nature of these which form the principal constituents of the wheat kernel have the property of imbibing water and forming gels. It is probable that in "dry" grain the water present is not sufficient to form a gel, i.e., the colloidal material does not have a continuous structure. Increasing the moisture content above the maximum at which discontinuity exists results in the formation of an elastic gel through which diffusion can occur. Further increases in moisture content up to maximum imbibition produce progressively less viscous gels. The rate of diffusion in a gel varies with the viscosity; in dilute gels diffusion takes place as in water, while in strong gels the rate is slower. Since the rate of respiration in grain depends in part upon the rate of diffusion between the various kernel structures, it follows that the less viscous the gelatinous material of which the cell contents are composed, the more rapid the rate of respiration.

Practical storage experiments conducted by Bailey showed the relation between the rate of respiration and the moisture content of wheat. A sample of wheat containing 15.5% of moisture stored in an elevator bin kept 333 days without heating sufficiently to necessitate turning it, while another lot containing 16.5% that was stored at the same time was heating in 49 days.

The effect of varying percentages of moisture upon the rate of respiration is shown in Table I and graphically presented in figure 2. In this and subsequent tables, the third column gives the number of calories of heat per 1000 grams dry matter per 24 hours. These figures are calculated according to the Milner factor of 2.6 calories per gram of respired carbon dioxide.

Increasing the moisture content from 12.50% to 14.78% resulted in an increased rate of respiration, but the rate is markedly accelerated when the percentage of moisture exceeds 14.78%. This serves to explain the increased tendency of grain to heat in storage when it contains in excess of the latter percentage of moisture.

Table I. Respiration of Minn. No. 169, bluestem wheat, from University Farm, incubated at 37.8° C for 4 days.

Moisture	CO_2 respired per 24 hours for each 100 grams dry matter	Calories of heat per 1000 g. D.M. for each 24 hours
Per cent	Milligrams	
12.50	0.54	0.014
13.93	0.65	0.016
14.78	0.86	0.022
15.42	1.62	0.042
16.08	2.88	0.072
16.65	6.86	0.178
17.07	11.72	0.304

Wt. per bushel of sample, 57 1/2 lbs. Wt. per 1000 kernels, 24.62 g.
Nitrogen on dry basis, 2.21%

The rate of acceleration of respiration as the moisture content increases is shown in Table 2. This data is based upon the interpolated values at even percentages of moisture, and the computed increase in respired CO_2 for each

increase of one per cent in moisture. These data are shown in Tables 20 and 21 in the appendix. In computing the data shown in Table 2 the following formula is employed:

$$\frac{K_m - K_{m-1}}{K_{m-1}}$$

in which K_m represents the respiration value at a particular percentage of moisture, and K_{m-1} represents the respiration value for the same wheat containing one per cent less of moisture.

It is evident from these data that the acceleration between 12 and 14 per cent of moisture is very gradual, while it increases markedly after 14 per cent is exceeded.

Table II. Acceleration of the rate of respiration of hard spring wheat with increasing moisture content.

$$\frac{K_m - K_{m-1}}{K_{m-1}}$$

Moisture	12-13%	13-14%	14-15%	15-16%	16-17%
Acceleration	0.160	0.172	0.662	1.407	3.018

Relation of the consistency of the wheat kernel to the rate of respiration.

The consistency of the wheat kernel serves to distinguish between two large groups, hard or vitreous, and soft or starchy. In general the hard, vitreous grain contains a higher percentage of gluten than does the soft, starchy wheat. The gluten content of the grain bears an important relation to the rate of respiration. Bailey has called attention to the difference in the water-imbibing capacity of the several colloids present in the wheat kernel, starch having only about one-fourth the imbibing capacity of wheat gluten. Other things

being equal, the viscosity of gel produced from a mixture of starch and gluten would vary directly with the percentage of gluten. Because of the relation of viscosity to rate of diffusion, and of the latter to respiratory activity, it would accordingly follow that with the same moisture content respiration would proceed more rapidly in a soft kernel than in a hard or vitreous kernel.

It was found that the rate of respiration was higher in soft red winter wheat, and materially higher in white winter wheat than it was in hard spring wheat. The respiratory activity at various moisture contents is shown in Tables III and IV. In Table V are shown the interpolated values in terms of CO_2 for even percentages of moisture respired by these three classes of wheat. The same data is shown graphically in Figure 3. From these data it appears that the quantity of heat evolved by hard spring wheat containing 14.5% of moisture, as evidenced by the rate of respiration, is evolved by soft red winter wheat containing 13.7% of moisture, and by white winter wheat containing 13.8% of moisture.

Table III. Respiration of soft red winter wheat, from Missouri, incubated at 37.8° C for four days.

Moisture	CO_2 respired per 24 hours for each 100 grams dry matter	Calories of heat per 1000 g. D.M. for each 24 hours
Percent	Milligrams	
13.07	0.65	0.014
13.63	0.80	0.021
14.70	0.95	0.025
15.45	2.00	0.052
16.37	5.06	0.132
17.40	22.03	0.573

Wt. per bushel of sample, 61 lbs. Wt. per 1000 kernels, 29.97 g.
Nitrogen on dry basis, 1.54%

Table IV. Respiration of white winter wheat, from Michigan, incubated at 37.8° C for 4 days.

Moisture	CO_2 respired per 24 hours for each 100 grams dry matter	Calories of heat per 1000 g. D. M. for each 24 hours.
Per cent	Milligrams	
11.94	0.48	0.012
13.04	0.60	0.015
14.32	0.89	0.023
15.57	3.20	0.083
16.83	22.77	0.590

Wt. per bushel of sample, 59 lbs. Wt. per 1000 kernels, 37.75 g.
Nitrogen on dry basis, 1.53%

Table V. Interpolated quantity of CO_2 respired per unit of time and material, at even percentages of moisture.

Class of 6 Wheat	CO_2 respired per 24 hours for each 100 g. D.M.					
	12% Moisture	13% Moisture	14% Moisture	15% Moisture	16% Moisture	17% Moisture
	mg.	mg.	mg.	mg.	mg.	mg.
Hard spring	0.50	0.58	0.68	1.13	2.72	10.73
Soft red winter		0.63	0.81	1.37	3.84	15.51
White winter	0.49	0.60	0.83	4.15	9.85	25.18

Relation of the plump and shriveled condition of the wheat kernel to the rate of respiration.

It is generally recognized that the velocity of enzyme action obeys in part the law of mass action. It accordingly follows that any factor which affects the quantity of either the substrate or the enzyme will cause variations in the respiratory activity of the wheat kernel.

In the development of the wheat kernel the shriveled condition is due generally to factors operating during the dough stage. The translocation of reserves is interfered with by rust, or some other agency, and results in the incomplete filling

of the endosperm. Accordingly to Brenchley[30] the germ portion of the kernel is developed earlier than the endosperm, and tends to escape injury from the agency causing shrunkness. The diminished size of the shrivelled kernel is therefore due principally to the decreased quantity of endosperm.

The diastatic activities of the kernel seem to be mainly invested in the germ. Mann and Harlan[31] found that the scutellum secretes the diastase and other enzymes concerned with the conversion of the endosperm reserves. This is indicated by the course of conversion. It may be reasoned by analogy that the respiratory enzymes are also largely confined to the germ portion. In the shriveled wheat germ the enzymes are represented practically as they are in the germ of the plump kernel. But there is about double the quantity of germ per unit of mass in the shriveled as compared with the plump grain. This is indicated by comparing the weight per 1000 kernels for the two wheats. Whereas shriveled wheat weighed 11.73 g. per 1000 kernels, plump wheat weighed 24.62 g. per 1000 kernels. Since this would result in an increased quantity of enzyme, the velocity of the reaction should proceed at a more rapid rate. That this is true is shown by the data in Table VI, and graphically in Figure IV. Tables 20 and 21, in the appendix, show the interpolated rates of respiration at even percentages of moisture, and the actual increase for each additional per cent of moisture.

These data show that the quantity of carbon dioxide respired by shriveled wheat is greater for all percentages of moisture than in plump wheat of the same class. Thus the quantity of CO_2 respired by plump wheat containing 14.5% of

moisture was respired by the shriveled wheat used when it contained only 12.8% of moisture. These data also show that the rate of respiration of shriveled wheat markedly increases when the moisture exceeds 14.8% of moisture, or one-half per cent lower than in case of the plump wheat. Like-wise, the value at higher percentages are practically doubled.

Table VI. Respiration of shriveled spring wheat, incubated at 27.6° C. for 4 days.

Moisture	CO_2 respired per 24 hours for each 100 grams dry matter	Calories of heat per 1000 g. D.M. for each 24 hours
Per cent	milligrams	
12.68	0.75	0.019
13.19	0.94	.024
14.29	1.38	.035
15.30	3.02	.078
15.68	4.50	.117
16.09	10.51	.273
16.44	16.92	.439
16.80	21.65	.561

Wt. per bushel of sample, 47 1/2 lbs. Weight per 1000 kernels, 11.73 g.

Nitrogen on dry basis, 2.03%

Table VII. Interpolated quantity of CO_2 respired per unit of time and material, at even percentages of moisture.

Class of Wheat	CO_2 respired per 24 hrs. for each 100 g. D.M.					
	12% Moisture	13% Moisture	14% Moisture	15% Moisture	16% Moisture	17% Moisture
	mg.	mg.	mg.	mg.	mg.	mg.
Plump spring wheat	0.50	0.58	0.68	1.18	2.72	10.93
Shriveled " "	0.65	0.88	1.26	2.54	9.41	22.65

Relation of the period of dampness of wheat to the rate of respiration.

At the time damp wheats are placed in store the excess moisture which is responsible for their dampness has been present

for varying lengths of time. This length of time is known for the purposes of this discussion as the period of dampness.

It appears from the foregoing discussion that in accordance with the law of mass action any factor which will quantitatively affect the ratio of enzyme to substrate will correspondingly affect the rate of respiration. In the case of shriveled wheat the proportion of enzyme is apparently increased, and an accelerated rate of respiration results. Similarly, any condition which increases the proportion of substrate would aslo accelerate the reaction.

In the discussion of the work of Milner and Gore, it was indicated that fats, proteins, or oils are utilized directly to a very slight extent by the respiratory enzymes. It is the carbohydrates which are mainly subject to physiological oxidation. Moreover, the ploysaccharides must first be hydrolysed to monosaccharides to render them available as substrate. The hydrolysis of starch through the activity of diastase in the presence of sufficient water, and at a suitable temperature, changes it to glucose, which, as has already been pointed out, is directly attacked by the oxidases.

It is evident from this that the length of time the excess water has been in contact with the kernel, will, other things being equal, bear a relation to the extent of starch hydrolysis and this in turn to the rate of respiration. A series of naturally damp wheats were collected by selecting samples taken from car lots by the State Grain Inspection Department. These were carefully cleaned, and their rate of respiration determines in the usual manner. These data are shown in Table VIII,

and graphically in Figure V. The increased quantity of glucose which has resulted from the hydrolysis of starch is probably responsible for the greater rate of respiration in these naturally damp wheats when they are compared with wheats that have been dampened but three days. From these data, as graphically presented, it is apparent that the curve of respiration of naturally damp wheat diverges from that of the freshly dampened wheat when the moisture content exceeds 13%. The rate of acceleration in the case of the naturally damp wheat is somewhat greater between 13 and 15% than is that of the freshly dampened wheat. The sharp break in the two curves occurs at about the same point, however, namely 14.7% to 14.8% of moisture. Furthermore, these two curves if extended would converge at 12% of moisture, indicating that the diastatic activity is much slower and the rate of substrate formation is reduced at this moisture content.

Table VIII. Respiration of natural hard spring wheat collected from carlots, and incubated at 37.8° C. for 4 days.

Lab'y. No.	Moisture	Wt. per Bushel	Wt.per 1000 Kernels	Nitrogen in dry Matter	CO_2 respired per 24 hours for each 100 grams D.M.	Calories of heat per 1000 g. D.M. for each 24 hrs.
	Per cent	Pounds	grams	per ct.	Milligrams	
G 128	12.47	62	30.56	2.14	0.61	0.015
G 127	13.11	63	32.94	2.16	0.75	0.019
G 124	14.70	58 1/2	24.60	2.05	1.49	0.038
G 120	15.51	52	26.92	2.31	3.26	0.084
G 104	15.73	59	28.64	2.47	3.94	0.102
G 103	16.00	59	25.72	2.37	5.69	0.147
G 107	16.53	56	22.88	2.25	10.65	0.275
G 109	16.90	51			13.86	0.358

Table IX. Interpolated quantity of CO_2 respired per unit of time and material, at even percentages of moisture.

Wheat	CO_2 respired per 24 hours for each 100 grams D.M.					
	12% Moisture	13% Moisture	14% Moisture	15% Moisture	16% Moisture	17% Moisture
	mg.	mg.	mg.	mg.	mg.	mg.
Freshly dampened wheat	0.50	0.58	0.68	1.13	2.72	10.73
Natural wheat	0.51	0.73	1.15	2.14	5.69	15.03

In order to substantiate the conclusion from the foregoing data an experiment was conducted to measure the effect of the length of time of storage at room temperature upon the rate of respiration. Two lots of wheat were dampened until they contained 15.21%, and 15.71% of moisture. These were stored at a temperature of about 25° C. for 55, and 108 days respectively. The respiration data from these stored samples are given in Table X, in contrast with the interpolated values at the same moisture content for freshly dampened wheat from the same lot. The quantity of respired carbon dioxide from wheat containing 15.21% of moisture which was stored for 55 days was about four times as great as that from the freshly dampened wheat of the same moisture content, while that from the sample containing 15.71% of moisture and stored 108 days was about eight times as great as for freshly dampened wheat of that moisture content.

Contrasting these data with that obtained from the naturally damp wheats shown in Table VIII, it appears that while the wheat containing 15.2% of moisture which was stored in the laboratory for 55 days respired 5.31 milligrams of CO_2 per 100 grams of dry matter, naturally damp wheat of the same moisture

content respired 2.59 milligrams of CO_2. The ratio of respired CO_2 from the naturally damp wheat containing 15.7% of moisture, and wheat of the same moisture content which was stored in the laboratory for 108 days, was 17.00 milligrams to 3.85 milligrams. In other words, there is a difference of from about two to four times in the rate of respiration of these two lots of stored grain. This difference can probably be attributed to the difference in the temperature at which the natural, and the dampened grain were stored, the former being exposed to the outdoor temperature during the winter months, while the latter were stored at the relatively warm temperature of 25° C.

Table X. Respiration of dampened wheat after storage at about 25° C. Incubated at 37.8° C. for four days.

	Lot A.	Lot B
Moisture, per cent	15.21	15.72
Number of days stored	55.	108.
CO_2 respired per 100 grams dry matter in each 24 hours, mg.	5.31	17.00
CO_2 respired per 100 grams dry matter in each 24 hours, mg.	1.35	2.17
CO_2 respired by naturally damp wheat from carlots, of same moisture content	2.59	3.85

Relation of the soundness of the wheat kernel to the rate of respiration.

It is apparent that any agency which will arrest the synthetic activities of protoplasm may result in the death of the latter. This disorganization or death of the protoplasm does not necessarily result in the simultaneous inactivation of

the hydrolytic enzymes which are concerned with the conversion of starch into available substrate for the oxydases. The disorganization of protoplasm under such conditions would result in renderning the grain kernel unsound and thus would affect its respiratory activity.

Wheat is often rendered unsound by frost. Different degrees of unsoundness are produced, according to the proportion of water in the grain at the time the freezing occurs. In the process of freezing the water in the moist kernel crystallizes out of the protoplasm, the death of which follows in consequence. The synthetic processes of the protoplasm cease, while the catalysis is subject to revival when the grain thaws out. Diastatic activities then become abnormally active, and in the presence of a high percentage of moisture considerable hydrolysis of the starch results. The monosaccharides thus accumulated serve as a substrate for the respiratory enzymes. The increased proportion of substrate serves to accelerate the respiratory activity through the operation of the law of mass action. That this is true is shown by the data in Tables XI, XII, and XIII, which show the relative rate of respiration of a lot of moderately frosted and of badly frosted wheat. The same data is shown graphically in Figure VI. Comparing the moderately frosted with the sound wheat, the respiratory values in case of the former are much higher than in the latter, amounting in certain instances to from two to three times as much. At 12.5% of moisture it respires at the same rate as sound spring wheat containing 14.5%. At 12%, 13%, and 14% or moisture the badly frosted wheat respired more rapidly than the moderately frosted grain.

Table XI. Respiration of moderately frosted wheat, incubated at 37.8° C. for four days.

Moisture	CO_2 respired per 24 hours for each 100 grams dry matter	Calories of heat per 1000 g. D.M. for each 24 hours.
Per cent	Milligrams	
12.32	0.74	0.019
13.38	1.04	0.027
14.44	1.89	0.049
14.95	3.75	0.097
15.42	5.21	0.153

Wt. per bushel of sample, 58 lbs. Wt. per 1000 kernels, 36.94 grams.

Nitrogen on dry basis, 2.02%

Table XII. Respiration of badly frosted wheat, incubated at 37.8° C. for four days.

Moisture	CO_2 respired per 24 hours for each 100 grams dry matter.	Calories of heat per 1000 g. D.M. for each 24 hours
Per cent	Milligrams	
13.79	1.63	0.041
14.67	2.64	0.067
15.74	5.24	0.136
16.81	9.82	0.254
16.95	11.40	0.296

Wt. per bushel of sample, 53 lbs. Wt. per 1000 kernels, 22.52 g.

Nitrogen on dry basis

Table XIII. Interpolated quantity of CO_2 respired per unit of time and material, at even percentages of moisture.

Wheat	CO_2 respired per 24 hours for each 100 g. D.M.					
	12% Moisture	13% Moisture	14% Moisture	15% Moisture	16% Moisture	17% Moisture
	mg.	mg.	mg.	mg.	mg.	mg.
Sound spring wheat	0.50	0.58	0.68	1.13	2.72	10.93
Moderately frosted	0.65	0.94	1.52	3.90		
Badly frosted	1.12	1.20	1.87	3.46	6.35	11.97

Certain of the wheat samples taken from car lots were frosted, and it was found that the rate of respiration in these frosted samples was materially higher than in the sound samples of the same moisture content. This is shown in Table XIV, which, in addition to the data concerning the character and performance of the frosted samples, contains in the last column the interpolated rate of respiration of sound samples of the same class and moisture content.

Table XIV. Respiration of several samples of frosted wheat from carlots, and containing varying percentages of moisture. Incubated at 37.8° C. for four days.

Lab'y. No.	Moisture	Wt.per Bushel	Wt. per 1000 Kernels.	Nitrogen on dry basis	CO_2 respired per 24 hours for each 100 grams dry matter	Interpolated respiration of sound wheat of same H_2O content.
		Pounds	grams	Per cent	Milligrams	Milligrams.
G 122	14.30	60 1/2	28.36	2.23	1.71	1.30
G 123	14.82	61	28.64	2.20	2.34	1.73
G 118	16.16	56 1/2	23.56	2.27	8.71	6.86
G 102	16.19	57	28.88	2.00	8.24	7.15

The relation of temperature to respiration.

It is known that temperature bears an important relation to the activity of enzymes. Pfeffer was among the first who subscribed to the view that increase in the intensity of respiration is concomitant with increase of temperature until the latter begins to influence injuriously all the vital processes. Ziegenbein[36] investigated the effect of temperature upon respiration, by measuring the amount of carbon dioxide excreted. His findings are given in Table XV. According to these data all temperatures above 45° are injurious, and the observed reduction

in respiration at 50° must be regarded as the result of the plants pathological condition owing to the excessive temperature. The diminution of the respiratory activity in the last two materials occurred at 40°. The increase in respiration near the maximum reaches, according to Clausen's[32] experiments on germinating wheat, as much as eleven times, and in the case of lupins, sixteen times the rate at 0° C.

Table XV. Results of Ziegenbein's determinations of the rate of respiration at different temperatures.

Material	Carbon dioxide respired at 10°	20°	30°	35°	40°	45°	50°	55°	60°
Potato tubers	1.17	2.22	4.62	7.85	10.24	12.22	11.14	10.30	2.7
Vicia faba (seedling)			55.2	78.72	65.1	57.8	20.80		
Abis excelso (shoots)			185.0	206.4	198.4	168.9	33.3		

Bailey[29] investigated the effects of air temperature, and the initial temperature of the grain, upon the rate of heating in storage. A lot of wheat containing 16.5% of moisture required from September 12 to October 31 to rise in temperature from 70° to 80° F., at total of 49 days, when the mean air temperature was 44.3° F. A similar lot containing the same percentage of moisture and stored on July 28 required only 11 days to rise from 70° to 80° in temperature, when the mean air temperature was 62.1° F. A lot of grain with an initial temperature of 70° F. took over five times as long to reach a temperature of 80° as another lot of the same moisture content but with an initial temperature of 74° F., or four

degrees higher. The latter observation particularly illustrates the acceleration of respiration with a rise in temperature.

According to thermochemical considerations, it is not the actual reaction but the velocity of the reaction that is influenced by the temperature. More specifically, temperature is one of the factors which determine the position of equilibrium between the substances taking part in a reversible reaction. In most of the enzymic processes, however, the equilibrium is only slightly dependent on the temperature. The velocity of reaction means the rate at which any system proceeds toward its equilibrium or final position. According to Van Hoff's early observations, in many cases, a rise of temperature of ten degrees doubles or trebles the velocity.

Another way in which temperature influences the enzymic reactions is by the destruction or inactivation of the enzyme itself.

The processes resulting from these two actions were first discussed by Tammann[33]. The assumption is made that the enzyme is inactivated in aqueous solution by a unimolecular reaction indpendently of whatever else is in the solution. From this a conclusion was drawn that the velocity with which the reaction proceeds at any time must be equal to the product of the amount of enzyme and the substrate still present.

The usual way of expressing the influence of temperature on an enzymic reaction is to give the reaction constants. The actual temperature curves of these reactions, however, differ from those of the ordinary chemical action, due to the dependence of

stability of the catalyst upon temperature. As the temperature rises, the increased velocity of the reaction is counterbalanced by the increasing inactivation of the enzyme. It has been suggested for this reason by Euler[34], that the temperature coefficient should be measured in a region where the destruction of the enzyme comes into consideration as little as possible.

These general principles are clearly brought out in the data presented in Table XVI, and the graphic representation of this data in Figure VII, which illustrates the usual course of the enzyme curve.

A large sample of Minn. No. 169 bluestem wheat from University Farm was dampened until it contained 14.96% of moisture. The minimize diastatic hydrolysis during the period of investigation the entire lot was placed in tightly sealed containers and kept in a refrigerator at a temperature of -8° to -13° C. The desired quantities were drawn from the refrigerator for incubation at the several temperatures, and the respired carbon dioxide determined in the usual manner. The moisture content of this lot was such as to give a fairly vigorous respiration.

The data in Table XVI show that the velocity of respiration increased to a maximum at 55° C. This temperature therefore is the one at which the most rapid production of heat would occur. A discoloration of the seed-coat of the wheat kernel begins to appear on some kernels at about 35°, while at 55° the whole mass is mahogany. At 65° the enzymes have been partly inactivated, while at 75° this inactivation has proceeded still

further, and some roasting of the grain has resulted.

Table XVI. Respiration of hard spring wheat containing 14.96% of moisture, and incubated at different temperatures.

Temperature		CO_2 respired per 24 hours for each 100 grams dry matter	Calories of heat per 1000 g. D. M. for each 24 hours.
Degrees C.	Degrees F.	Milligrams	
4	39.2	0.24	0.006
25	77.0	0.45	.011
35	95.0	1.30	.033
45	113.0	6.61	.171
55	131.0	31.73	.824
65	149.0	15.71	.408
75	167.0	10.28	.265

In the following table is shown the proportional increase for each ten degrees rise in temperature. It is apparent from these figures that the quantity of CO_2 has doubled between 4° and 25°. An increase from 25° to 35° has resulted in nearly trebling the rate, while between 35° and 45° it has increased five times. The data in this table is obtained by employing the formula $\frac{V_t + 10}{V_t}$ in which V_t represents the rate of respiration at the specified temperature, and $V_t + 10$ represents the rate at a temperature ten degrees higher. The values at 5° and 15° were obtained by integrating the actual respiratory data obtained at 4°, 25° and 35° C.

t	$\frac{V_t + 10}{V_t}$
5°	1.16
15°	1.55
25°	2.89
35°	5.08
45°	4.80
55°	0.49

In Table XVII the above ratios are compared with those found by Slator in studying the temperature coefficients of alcoholic fermentation by living yeast. Slator's[35] data show a decreased rate of acceleration as the temperature rises, which is exactly the reverse of a similar comparison of the acceleration of the complex phenomenon known as respiration. This may be explained by the fact that in respiration of the wheat kernel there are at least two processes operating simultaneously: the production of substrate, i.e., the hydrolysis of starch by diastase, and the oxidation of the hydrolysed starch by respiratory enzymes. A rise in temperature accelerates the former process and thus produces an accumulation of substrate. In accordance with the law of mass action this would increase the velocity of the reaction caused by the oxidases. The oxidases are simultaneously oxidizing this substrate at an increased rate.

Table XVII. Comparison of the rate of acceleration of yeast formation as reported by Slator, with that of respiration.

t	$\frac{V_t + 10}{V_t}$	
	Yeast Fermentation	Respiration
5°	5.6	1.16
10°	3.8	- -
15°	2.6	1.55
20°	2.25	- -
25°	1.95	2.89
30°	1.60	- -
35°	- -	5.08
45°	- -	4.80

Respiration in oxygen-free medium.

In normal metabolism of the green plant the respiratory

gaseous exchange is far less intense than the assimilatory gaseous exchange. In the vital phenomena of the seed, however, the respiratory exchange is infinitely greater than the assimilatory exchange. Oxygen is drawn from the air as well as from dis integration of reserve materials and carbon dioxide is excreted.

In case of seeds which are respiring under favorable conditions of moisture and temperature, it is apparent that the supply of free atmospheric oxygen may become depleted, and the respiratory enzymes may activate the inert oxygen in the molecule of reserve materials such as carbohydrates. Respiration under such conditions is known as intra-molecular respiration. The quotation from Pfeffer's views upon the relationship that exists between aerobic and intra-molecular respiration may well be repeated at this point. Pfeffer[9] maintained that intra-molecular respiration succeeds normal respiration when oxygen is deficient; decomposition of organic bodies is the primary phenomena in both processes. The decomposition must result in the formation of an oxidizable body, which, in the presence of free oxygen, takes it up, but which, in its absence satisfies its requirements so far as oxygen is concerned, from other compounds containing it. The evolution of CO_2 in the absence of oxygen had been studied by Rollo as early 1798, and by De Saussure in 1804. Since that time the relation of oxygen to the living organism has been the subject of considerable study.

To determine the effect of the depletion of oxygen upon the respiration of wheat, samples of two different moisture

contents were placed in cylinders, and the air was completely replaced by nitrogen. These samples contained 15.6% and 17.6% of moisture respectively and they were incubated for 4 days at 23.9° C. The comparative rate of respiration in this oxygen-free and in normal atmosphere is given in Table XVIII and Figure IX. This shows that in case of 15.6% of moisture, the ratio of the quantity of CO_2 respired in the absence of oxygen to that respired in a normal atmosphere is 1: 2.56, while in case of the sample containing 17.6% of moisture it is 1: 2.43.

Table XVIII. Comparative rate of respiration in oxygen-free and normal atmosphere. Incubated 4 days at 23.9°

Moisture	CO_2 respired per 100 g. dry matter in each 24 hours. Oxygen-free Atmosphere	Normal Atmosphere
Per cent	milligrams	milligrams
15.6	0.43	1.10
17.6	2.80	6.80

Influence of accumulated carbon dioxide upon respiration.

According to the conclusions of some of the earlier investigators, CO_2 exerts an injurious effect upon respiration. In the writer's studies the respiring grain was sealed in the respiration chamber so that there was no communication with the outside atmosphere. The respired CO_2 accordingly accumulated in the chamber. The more favorable the conditions of temperature and moisture, the greater the quantity of CO_2 which was thus accumulated.

A series of experiments were conducted to determine the effect of this accumulated carbon dioxide upon the rate of respiration from day to day. A sample of clean, natural wheat containing 15.05% of moisture was selected for this purpose. This was divided into a number of portions of 500 grams each, which were sealed into the cylinders and incubated at 37.6° C. Each day for 4 days a cylinder was removed, and the CO_2 determined in the usual manner. After this time one cylinder was removed every second day. Dividing the first 12 days into three periods of four days each, it is shown in Table XIX and Figure VIII that the average rate during the first 4 day period is materially greater than during the second and third periods. The table also shows that the rate the first day was considerably greater than the average rate per day for the first 4 day period.

Table XIX. Rate of respiration per day for several successive periods.

Period	CO_2 respired per 100 grams of dry matter.
	Milligrams
First day	4.11
Average rate per day for first 4 day period	2.68
" " " " " second " " "	1.49
" " " " " third " " "	1.11

CONCLUSIONS

Determination of carbon dioxide furnishes an accurate index to respiratory activity, and can be computed in terms of heat evolved when an elaborate respiration calorimeter is not available.

Truog's method for the determination of carbon dioxide with modifications satisfies the requirements of plant respiration studies, in that it makes possible the rapid removal of accumulated carbon dioxide from the respiration chamber, and is further capable of accomodating wide variations in the quantity of the gas.

Distribution of water added in dampening wheat is complete in less than 72 hours, as shown by determinations of the swelling of the grain. This made it possible to measure the rate of respiration at various moisture contents after the grain had been deampened for 72 hours.

Moisture is one of the determining factors in respiration. It establishes different rates of diffusion between various structures of the wheat kernel, and therefore any gain in the moisture content of the grain increases its respiratory activity. The acceleration is gradual and fairly uniform until the percentage of moisture exceeds 14.7% in the case of plump hard spring wheat, when it is very marked.

Density of the wheat kernel general parallels the gluten content. Gluten possesses the property of imbibing more water than starch, and thus varying percentages of gluten result in varying degrees of viscosity at the same moisture content.

The relative viscosity in turn affects the rate of diffusion, which directly affects the rate of respiration. The soft, starchy wheats thus respire more rapidly than hard, vitreous wheats.

Plumpness of the wheat kernel affects the rate of respiration, as shown by contrasting the respiration of plump and shriveled wheats. The shriveled wheat respired two to three times as much as the plump wheat at moisture contents above 14%. At percentages of moisture below 14% the difference is not very marked. The high acceleration of respiration in shriveled wheat containing more than 14% of moisutre is attributed to the larger proportion of enzyme to substrate as compared with plump wheat.

Period of dampness, i.e., the length of time the excess moisture has been present in the wheat, bears a relation to the rate of respiration. This is shown by comparing the respiration of freshly dampened wheat with that of naturally damp grain, and with grain that had been dampened and stored for varying lengths of time. The curve of respiration diverges from that of freshly dampened wheat when the moisture content exceeds 12%, and this divergence is more marked after 13% is reached. In case of wheat dampened and stored the larger quantity of CO_2 respired varies directly with the number of days the wheat remained in storage. The temperature at which the grain is stored affects the rate of diastatic action, as indicated by the higher rate of respiration of wheat stored at warm temperatures than that stored at the outdoor temperature during the winter months.

Unsoundness of the wheat kernel, particularly as caused by freezing of the unrkpe grain, results in higher respiratory

activity. This was shown by comparing moderately and badly frosted wheats with sound wheat. The frosted wheat respired more rapidly than the sound, the rate varying directly with the degree of frost damage. It was occasioned by the arrest of the synthetic processes on freezing, and subsequent activities of the hydrolytic enzymes on thawing of the frozen wheat. The accumulation of glucose as a result of starch hydrolysis furnishes larger quantities of substrate to the respiratory enzymes.

Increasing temperature accelerates the rate of respiration until 55° C. is reached. As the temperature rises the diastatic action upon starch increases. A point is reached however, at which the enzyme activity diminishes. The rate of respiration at different temperatures is logarithmic in form.

Intra-molecular respiration takes place when wheat respires in oxygen-free atmosphere. The ratio of the rate of respiration in such an atmosphere to that in a normal atmosphere is about 1: 2.5

Accumulation of carbon dioxide in the respiration chamber decreases the rate of respiration. The mean rate by four day intervals is highest for the first 4 days, and therefore, this length of time was made the respiration period in all cases.

APPENDIX.

Table XX. Interpolated quantity of CO_2 respired per unit of time and material at even percentages of moisture.

	CO_2 respired per 24 hours for each 100 grams D. M.					
	12% Moisture	13% Moisture	14% Moisture	15% Moisture	16% Moisture	17% Moisture
Hard spring	0.50	0.58	0.68	1.13	2.72	10.93
Soft red winter	- -	0.63	0.83	1.37	3.84	15.51
White Winter	0.49	0.60	0.81	2.15	9.85	25.18
Shriveled spring	0.50	0.88	1.26	2.54	9.41	22.65
Moderately frosted spring	0.65	0.94	1.52	3.90		
Badly frosted spring	1.12	1.20	1.87	3.46	6.35	11.97

Table XXI. Acceleration of the rate of respiration with increasing moisture content.

Type of wheat	$\frac{K_m - K_{m-1}}{K_{m-1}}$				
	12-13% Moisture	13-14% Moisture	14-15% Moisture	15-16% Moisture	16-17% Moisture
Hard spring	0.160	0.172	0.662	1.407	3.018
Soft red winter	- -	.318	.650	1.803	3.039
White winter	.225	.350	1.654	3.580	1.557
Shriveled spring	.354	.432	1.016	2.705	1.407
Moderately frosted spring	.446	.617	1.566		
Badly frosted spring	.071	.558	.850	.835	.885

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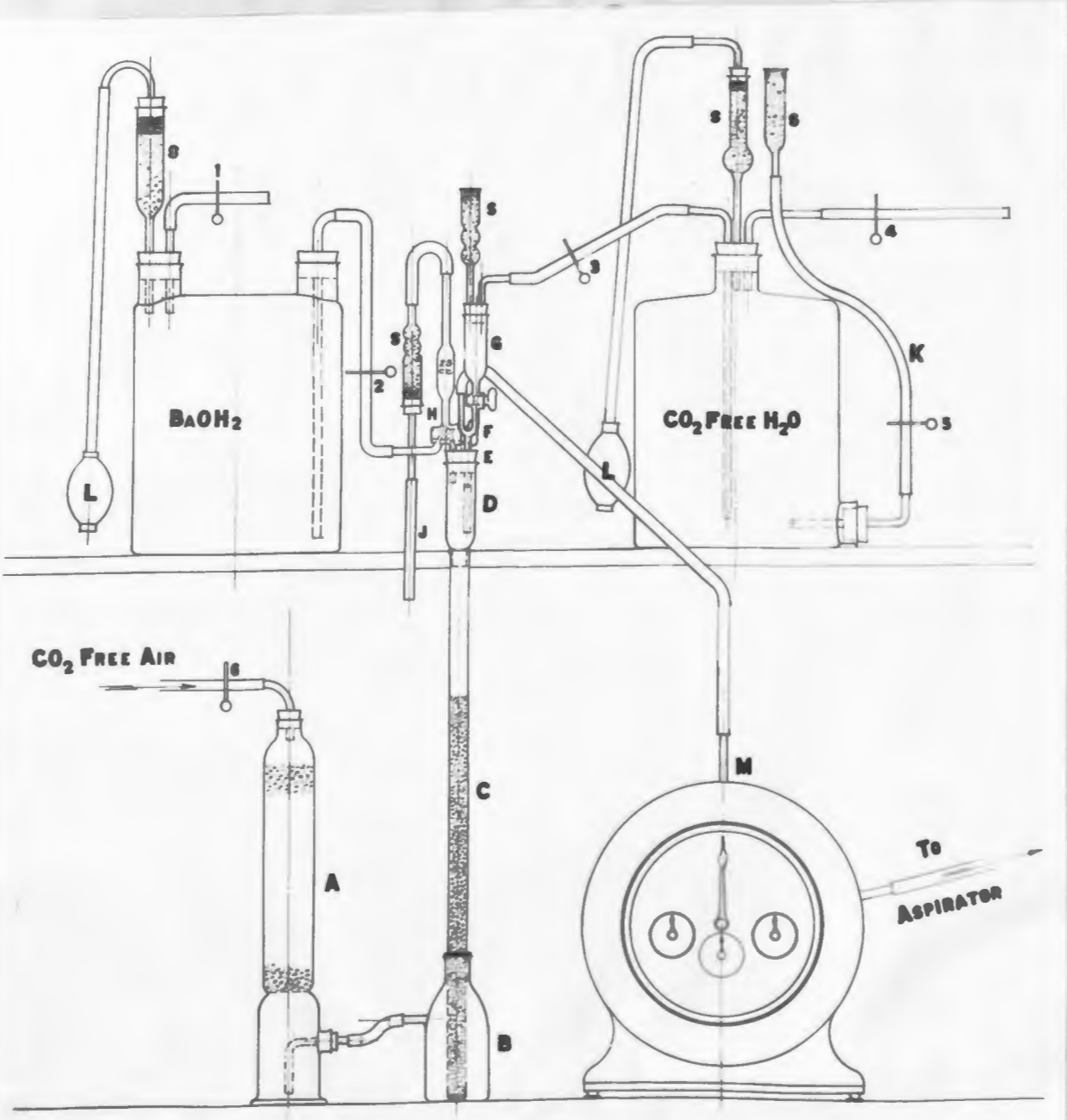
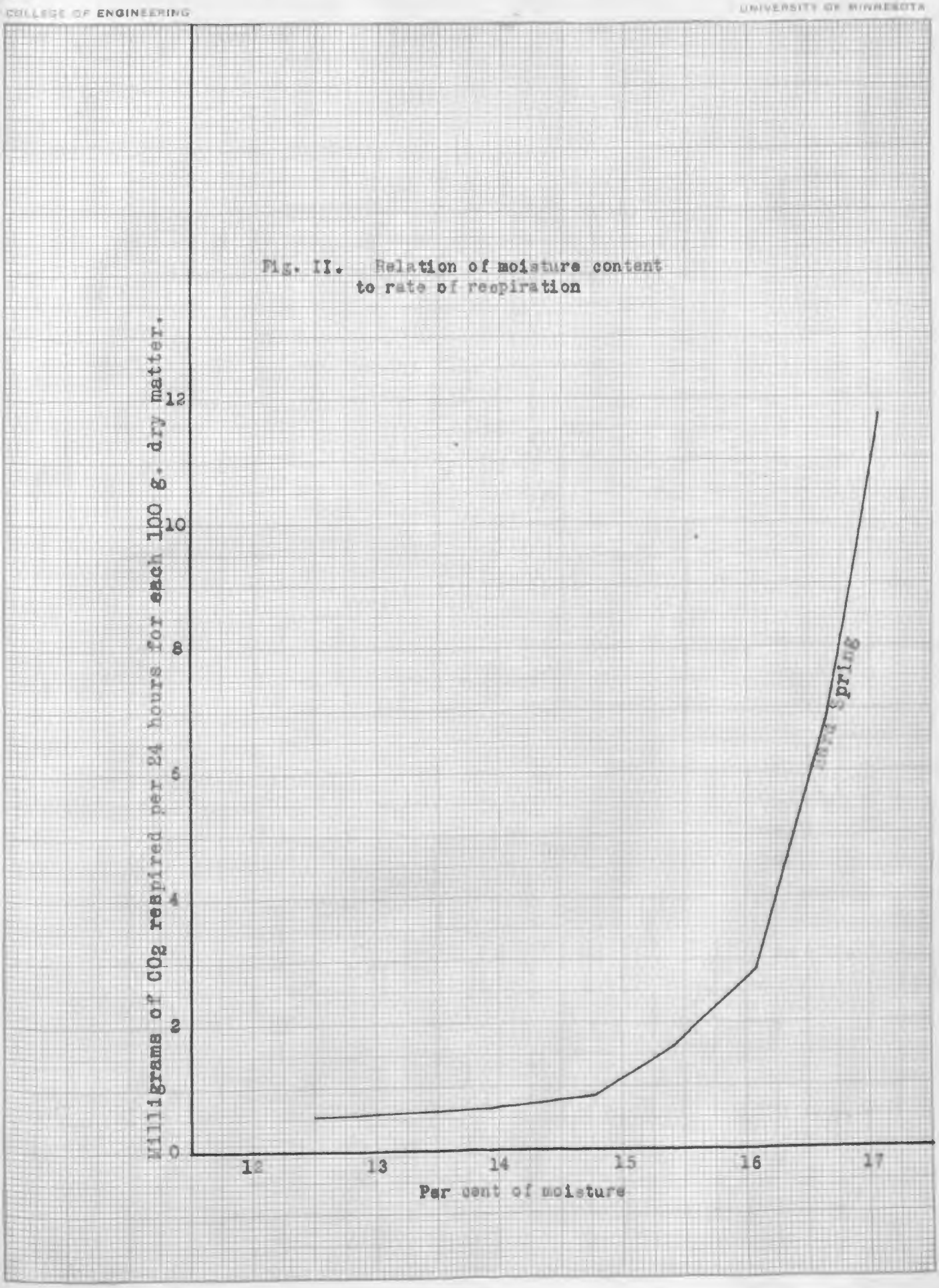
Figure I.



Modification of Truog's Tower for the determination of carbon dioxide.

Fig. II. Relation of moisture content to rate of respiration



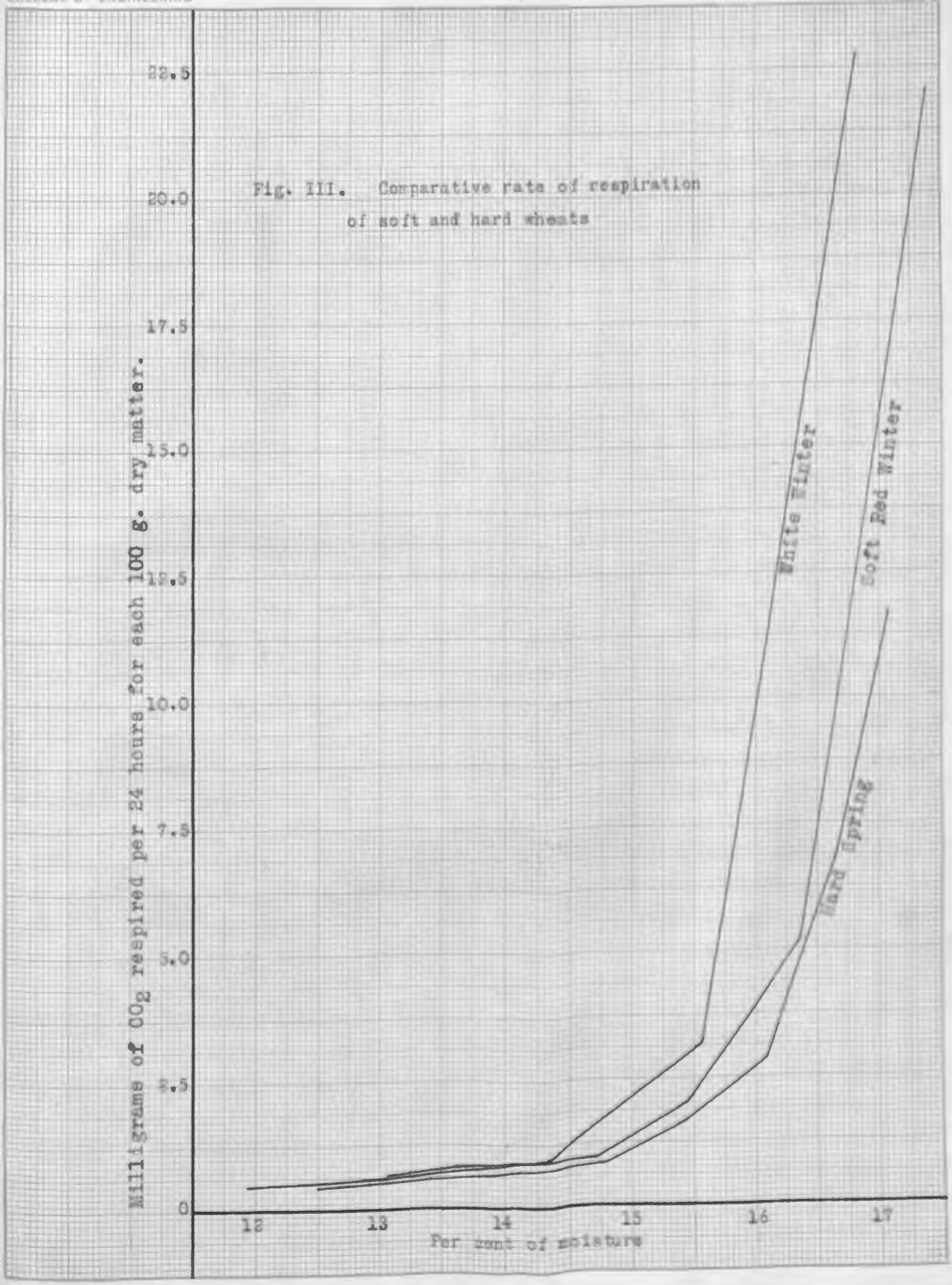


Fig. III. Comparative rate of respiration of soft and hard wheats

Fig. IV. Comparative rate of respiration of shriveled and plump wheats

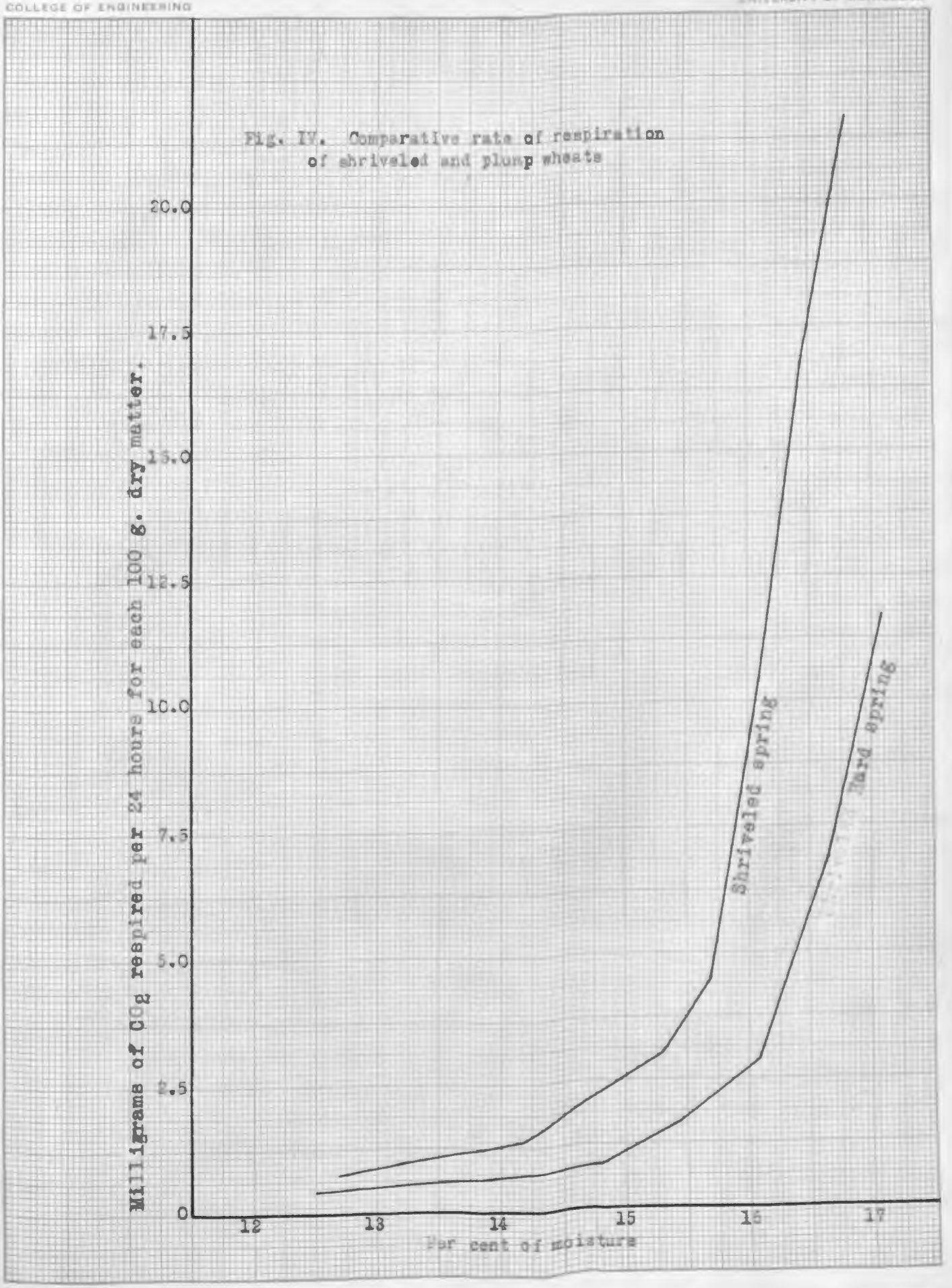


Fig. V. Rate of respiration of natural and dampened wheats.

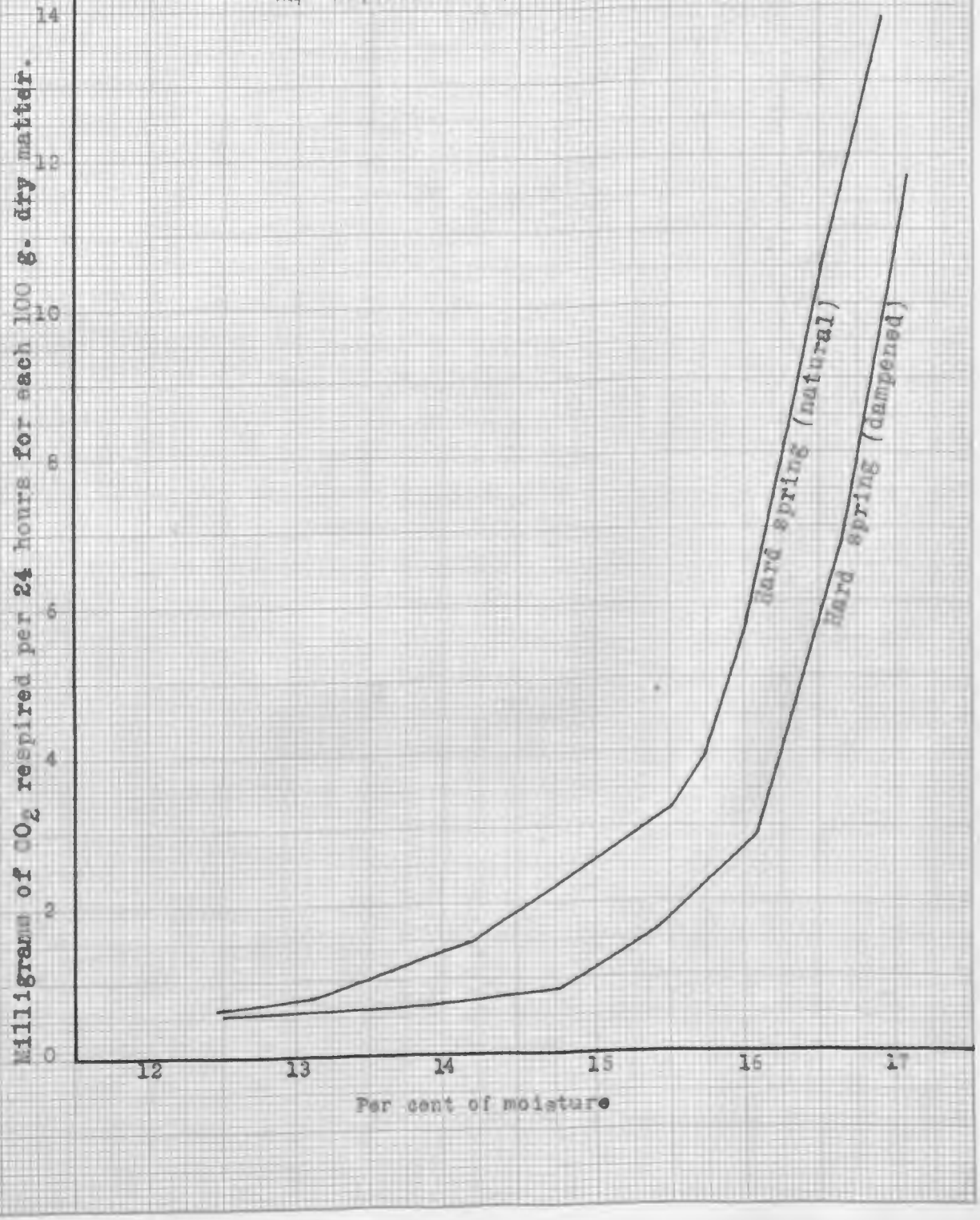


Fig. VI. Comparative rate of respiration of frosted and sound spring wheat

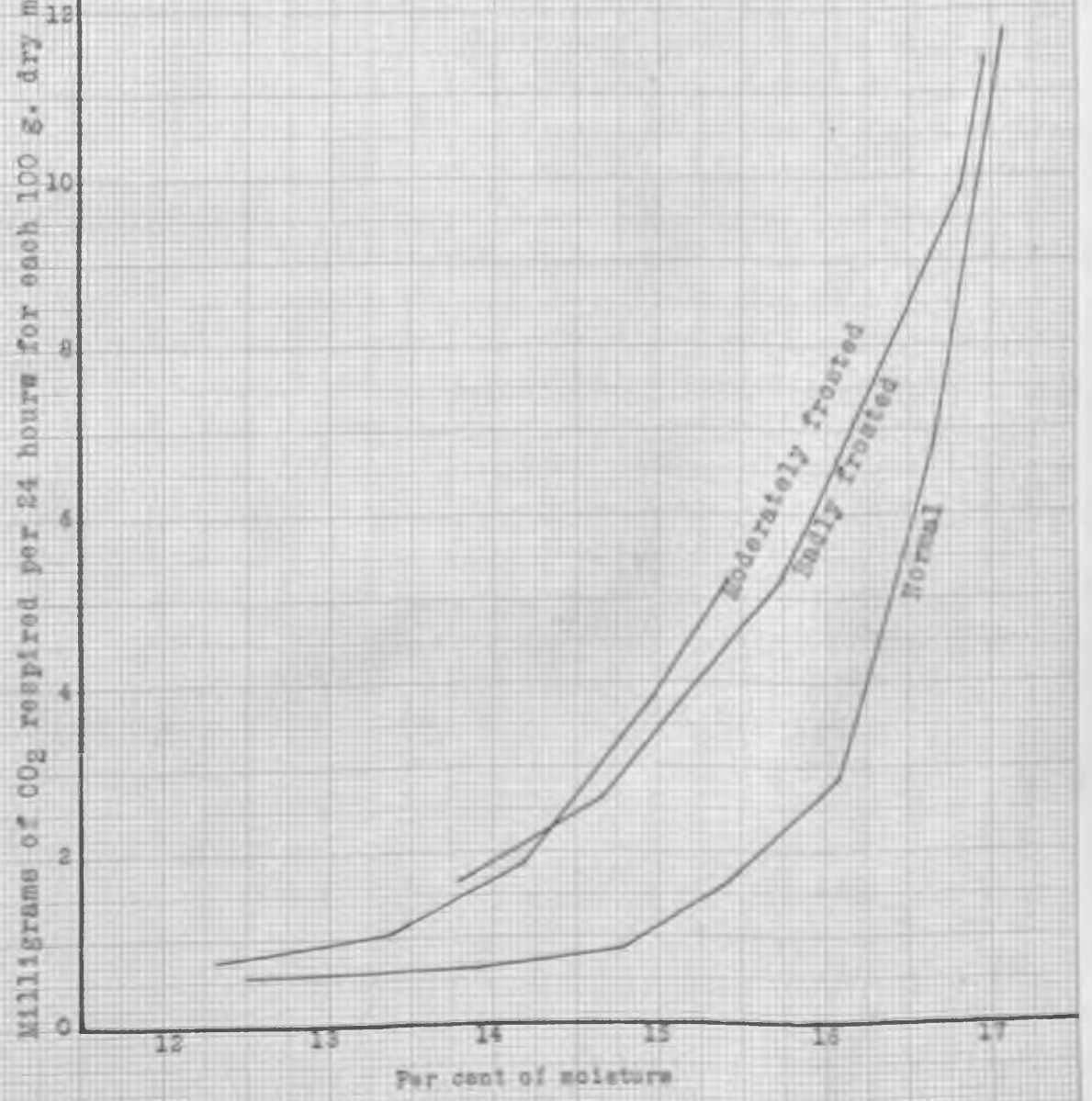
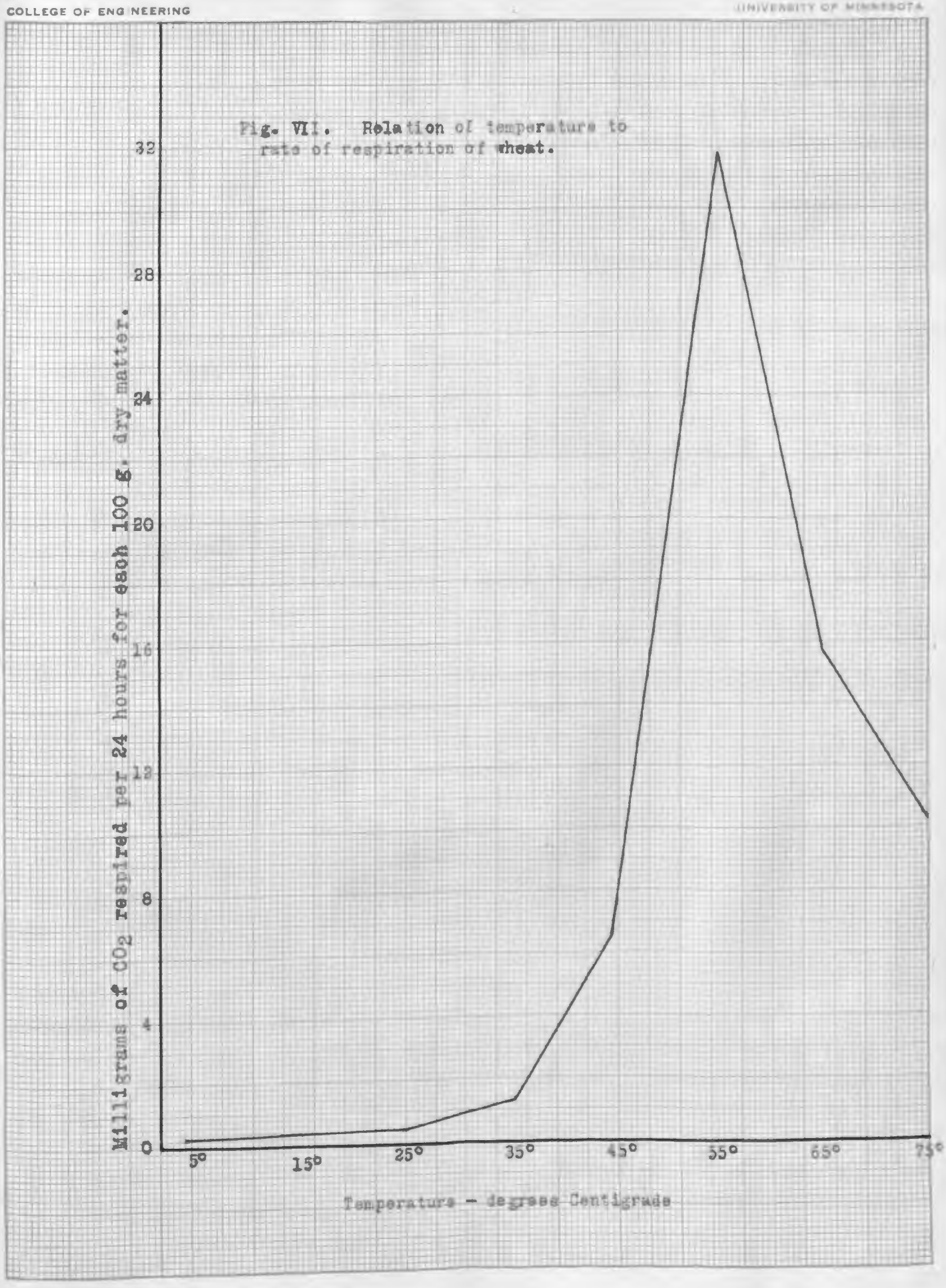


Fig. VII. Relation of temperature to rate of respiration of wheat.



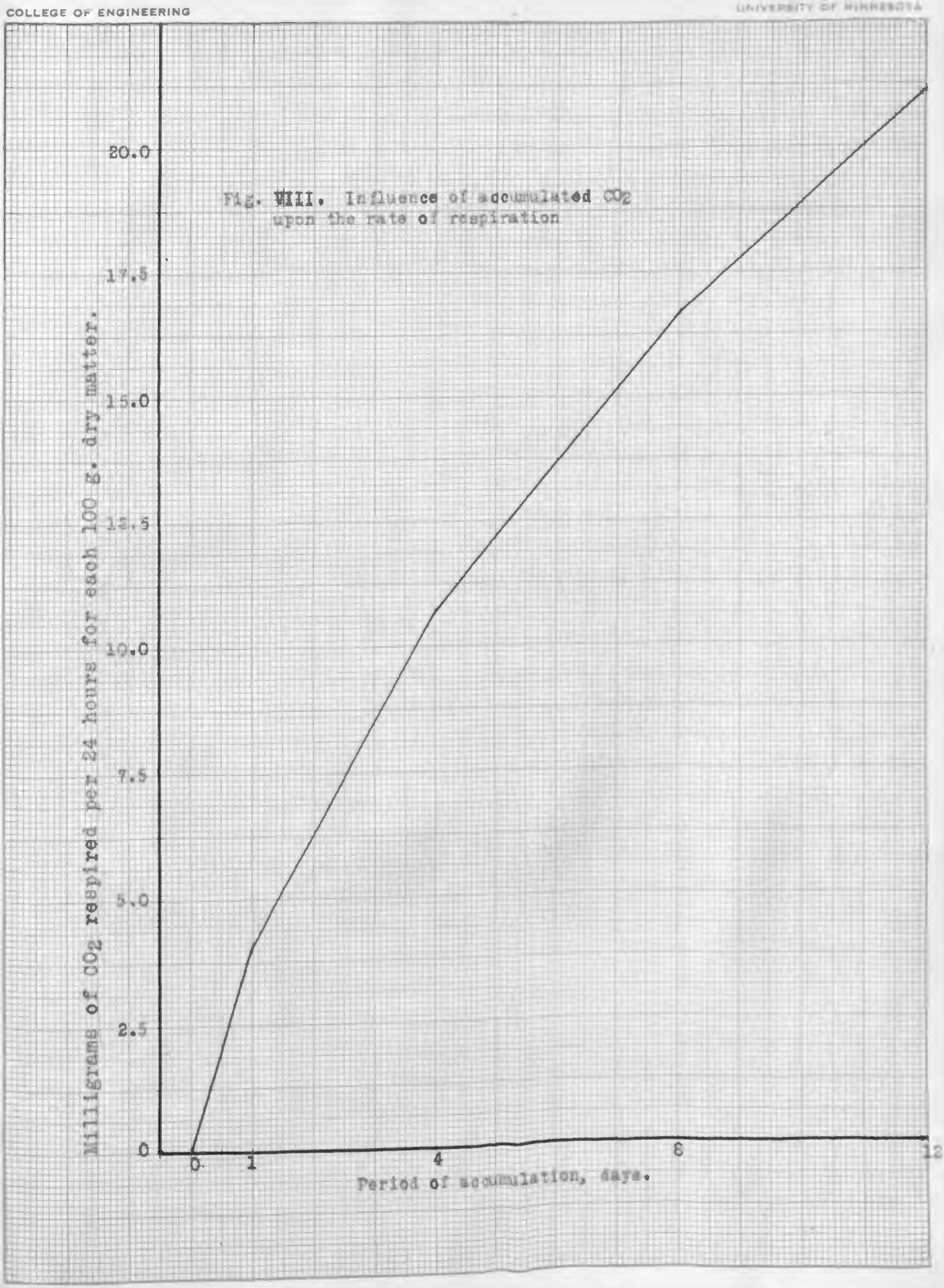


Fig. VIII. Influence of accumulated CO_2 upon the rate of respiration

Fig. IX. Comparative rate of respiration in oxygen-free and normal atmosphere