AN X-RAY STUDY OF THE CHANGES IN THE STRUCTURE
OF WHEAT STARCH IN STALING BREADS

by

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INTRODUCTION

Katz, et al. (5, 6, 7), Miller, et al. (11) and Hellman, et al. (4) have suggested that the staling property of bread is associated with a change in the crystalline property of the starch component of the flour used in making the bread. Katz showed by x-ray powder diffraction diagrams that the structure of the bread changed from an amorphous or non-crystalline state in freshly baked bread to a crystalline state during the staling process and attributed these structural changes to the starch component of the bread. The diffraction patterns of these various structural modifications were designated by Katz as "A", "B" and "V". These modifications will be discussed in detail later in the discussion of starch properties. Katz found that the crystalline organization which exists naturally in the starch of wheat flour and dough, termed the "A" type organization, disappeared during the baking process. The bread became largely amorphous in structure with a few weak rings appearing on the diffraction pattern termed a "V" pattern. As staling progressed, another crystalline form appeared which was identified by its diffraction pattern as a "B" modification. The "A" modification did not reappear.

Hellman, et al. (4) prepared starch gels by heating mixtures of starch of the "A" modification with water. The crystalline organization of the starch disappeared or at least was reduced in extent during the heating process. The gels were

then stored under conditions which prevented moisture loss.
During storage the starch molecules reordered and exhibited
crystalline x-ray diffraction patterns. Gels whose moisture
content was less than the moisture content comparable to that
of bread reordered to give back the "A" type crystallinity.
The gels whose moisture content was comparable to or greater
than that of bread reordered to give the "B" type crystal-
linity.

It is apparent from the above that changes in the struc-
ture of bread as it stales are connected with the changes in
crystallinity of the starch contained in the bread. The pres-
ent study was undertaken in an effort to determine quantita-
tively the changes which take place in the crystallinity of
starch in bread during the staling process using x-ray tech-
niques. It might be expected that a change in crystallinity of
starch could be measured by determining the change in dimen-
sions of the unit cell of the starch crystal. It should also
be of interest to attempt to observe the mechanism of growth of
the starch crystals in staling bread.

It is known that certain enzymes retard the staling proc-
ess (Miller, et al., 11). If the staling property is actually
a recrystallization process in the starch component of the
bread, then it might be expected that an investigation of bread
treated with these enzymes would show some effect on the re-
crystallization of the contained starch. Before discussing the
experimental procedure involved in this investigation, it would

be helpful at this point to make a preliminary study of the literature concerned with starch and its properties.

Starch is a high polymeric carbohydrate containing glucose as the repeating unit. The monomeric glucose units are joined by glucosidic oxygen linkages in two structurally different substances (Kerr, 8).

The amylose fraction (22% - 26% of whole starch) (Whistler and Smart, 16), one of the substances of starch, is thought to consist of linear chains of glucose residues united by 1-4, α-linkages in which the glucosidic oxygen bonds project from the glucose ring on the same side as shown schematically in Plate I, Fig. 1. Cellulose, on the other hand, consists of chains of glucose residues joined in 1-4, β-linkages in which the oxygen bonds project to opposite sides of the ring in successive glucose units as shown in Plate I, Fig. 2. The other structural substance of starch is amylopectin (78% - 74% of whole starch) (16) which contains a large proportion of 1-4, α-linkages as does amylose but periodically, approximately every 20 glucose residues, linearity is interrupted by an irregular 1-6, linkage. This linkage serves to connect other chains of glucose units to the primary chain thus creating a complex branched structure as shown schematically in Plate I, Fig. 3.

Katz and van Itallie (6) showed that raw starch exhibits a crystalline organization as evidenced by x-ray powder diffraction diagrams. They showed further that native starches may be grouped into three general classes from consideration of these

EXPLANATION OF PLATE I

Fig. 1.   Section of amylose molecule.

Fig. 2.   Section of cellulose molecule.

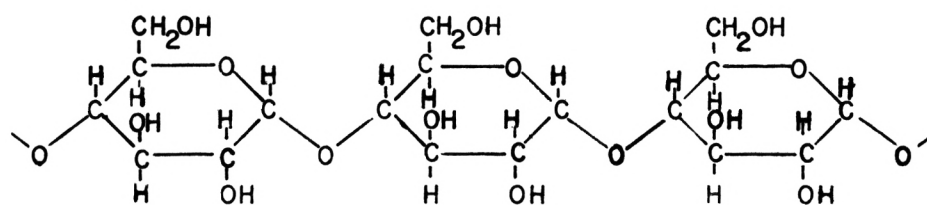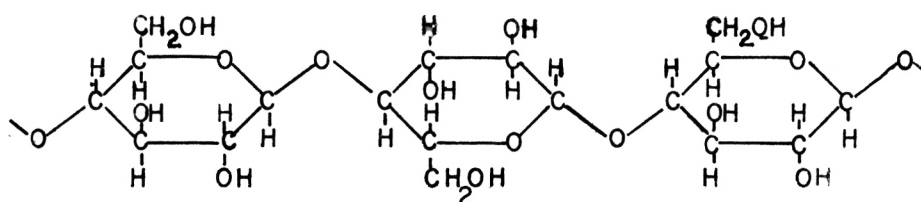Fig. 3.   Section of amylopectin molecule.

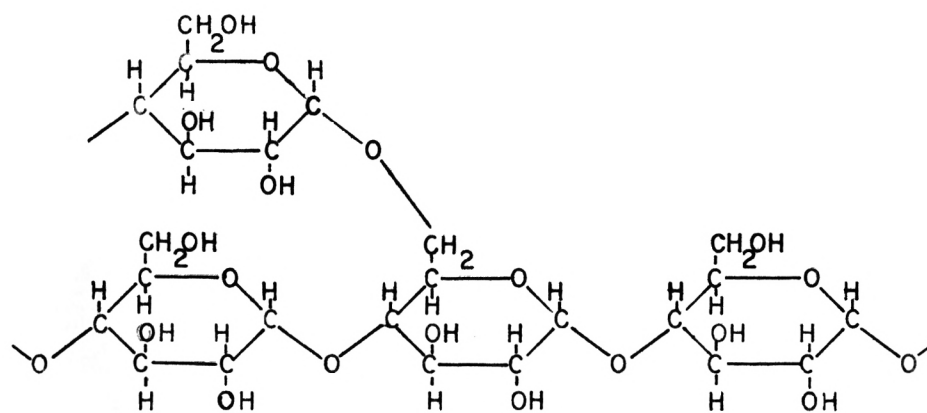PLATE I

Fig. 1.

Fig. 2.

Fig. 3.

powder diagrams. Starch from the seeds of the Graminee family such as wheat, corn, barley and rice all exhibited identical powder diagrams designated by Katz as an "A" diffraction diagram. Potato and other tuber starches exhibited a somewhat similar but yet distinctly different pattern designated as a "B" pattern. Starch from arrowroot, tapioca and real sago gave a "C" pattern which appeared to be intermediary between an "A" and a "B" pattern. A fourth pattern designated as a "V" pattern was observed in diffraction diagrams of freshly baked bread and in diffraction diagrams of starch gels which had been precipitated by alcohol.

Katz identified six diffraction rings from the x-ray powder patterns of native starch. The rings were numbered from one to six with the ring having the smallest diameter (indicating diffraction from the largest interplanar spacing) designated as the first ring. The characteristics of the three types of powder patterns are as follows:

No. 1 ring absent, No. 6 ring single: "A" pattern.

No. 1 ring present (strong intensity), No. 6 ring double: "B" pattern.

No. 1 ring present (lower intensity than in "B" pattern), No. 6 ring single: "C" pattern.

The combination with the No. 1 ring absent and the No. 6 ring double was not observed.

The No. 3, No. 4 and No. 5 rings were of the same diameters and relative intensities in the patterns of the three starches.

The "V" pattern apparently bears no simple relation to the other three types. It is characterized by three diffraction rings which are sometimes slightly broad.

Later investigations have shown that the crystalline properties of whole starch can be attributed solely to the amylose fraction. The highly branched structure of amylopectin displays an amorphous structure.

Bear and French (1) analyzed powder patterns of both the "A" and the "B" modifications of starch in an effort to compare in detail the observed similarities in the gross structure of the two types. They employed corn starch as a source of "A" diffraction patterns and potato starch as a source of "B" patterns. They showed that all of the diffraction rings of the powder patterns were caused by the single crystalline amylose component of starch. Starch samples which were specially purified in order to remove fatty and other materials yielded powder patterns identical to those prepared without special treatment. Other samples prepared with equal parts of starch and water were subjected to autoclaving at 120° C., let stand for 24 hours at various controlled temperatures and finally dried in air at these temperatures. The starch recrystallized during the period of standing and drying and gave good x-ray powder diffraction patterns. Those samples dried below 50° C. yielded patterns resembling the "B" type pattern; those at 50°C. and above exhibited chiefly the "A" type pattern. It was possible to obtain all intermediate stages of the transition between the pure "A" and "B" types in the range of temperatures studied. They concluded that in going from an "A" to a "B" modification relatively slight alterations in unit cell dimensions are nec-

essary, and this relationship is independent of the validity of
any unit cell which may be proposed for the starch crystal.
The unit cell may be defined as the smallest possible subdivi-
sion which, by the repetition or translation of itself in all
directions, builds up the crystal (Clark, 3). The proposed
unit cells which follow were indexed from powder pattern data
from starch samples. Linear dimensions are in Angstrom units.
The proposed unit cells each contain four glucose residues.

Table 1. Unit cell dimensions of the "A" and "B" starches
prepared by Bear and French (1).

Starch modification	a_0	b_0	c_0	α	β	γ	Volume $(\text{Å})^3$
"A"	15.4	8.87	6.18	87.0°	86.9°	92.8°	843
"B"	16.1	9.11	6.34	90.0°	90.0°	90.0°	930

X-ray powder patterns yield little insight into the basic
structure of crystalline materials of low order symmetry. For
crystals of this type such as starch, fiber patterns are neces-
sary to more completely designate the structure of the unit
cell. In contrast to some other carbohydrates such as cellu-
lose and chitin, starch is not deposited in living organisms as
fibers whose crystallites are oriented parallel to one another.
Its microscopic grains actually possess a radial orientation of
crystallites. Thus, x-ray diffraction from even a single whole
grain yields a powder pattern like that obtained from powders of

crystalline particles oriented at random. Efforts to prepare starch fibers from whole starch generally have been unsuccessful due to the branched structure of the whole starch. The resulting fibers were too short even when stretched to give x-ray fiber patterns by ordinary methods.

Rundle, et al. (12) prepared thin films of the unbranched amylose component of whole starch. Stretching these films gave a satisfactory fiber orientation to the crystallites. From the x-ray patterns of these fibers they have proposed a unit cell indexed as orthorhombic with the axes in the basal plane of 16.0 A. U. and 9.2 A. U. and a fiber axis of 10.6 A. U. perpendicular to the basal plane. Powder patterns of the amylose films proved to be identical to the "B" modification of starch. A study of experimental and theoretical mass densities indicated that there must be eight glucose residues per unit cell. These workers proposed a stretched chain configuration of the amylose molecule with two glucose residues per period, thus requiring that there be four chains running through the unit cell. The amylose films were highly birefringent and in convergent polarized light they showed a uniaxial, optically negative interference pattern similar to the fibers of cellulose and protein. The dimensions of their proposed unit cell are similar to those of the chitin unit cell except that the 16 A. U. spacing is increased to 19 A. U. in chitin (Meyer and Pankow, 10). In chitin the glucose residues are arranged with the planes of the glucose rings nearly parallel to the 19 A. U. axis and the

normals to the rings approximately parallel to the 9 A. U. axis. By analogy with the chitin unit cell, these workers proposed that the four chains run through the unit cell of amylose in such a way that the planes of the glucose rings lie parallel to the 16 A. U. axis. They stated that there are two space groups possible: D_2^1-P222 and D_2^2-P22$_1$2. The first requires that there be a two-fold axis parallel to the chains, the second specifies that there be a two-fold screw axis in this direction. In either case the two-fold axes normal to the chains must run between the chains, and in either case half the chains will be running in one direction, and half in the other. Plate II, Fig. 1, shows the model for the D_2^1-P222 space group. They stated that the chains must be unequally spaced along a_o and c_o since the odd-ordered reflections (h00), (00ℓ), (h0ℓ) were observed to be quite intense.

Kreger (9) has succeeded in obtaining x-ray fiber patterns of type "B" starch by irradiating a small sector of a single starch grain with a micro-beam of x-rays of the order of ten microns in diameter. The small sector thus irradiated showed small angular dispersion in orientation of the crystallites and thus yielded a pattern which could be indexed as a fiber pattern. Kreger used relatively large (approximately 80 microns in diameter) starch grains from the pseudo-bulb of the orchid Phajus grandifolius. Powder patterns of the Phajus starch grains showed identity with the "B" modification of starch.

The unit cell of the starch crystal was indexed provision-

ally on the basis of an orthorhombic cell with axes in the basal plane 9.0 A. U. and 15.6 A. U. in fair agreement with those reported by Rundle, et al. The fiber axis was found to be 10.6 A. U. in exact agreement with the fiber axis reported by Rundle, et al.

Kreger found it difficult to accept the stretched chain configuration containing two glucose residues per period proposed by Rundle, et al. because of difficulties due to the α-linkage of the glucose residues. He pointed out, as did Rundle previously (12), that one would expect a similar chain to be far more crumpled, resulting in a much shorter fiber period than the one actually found. Kreger showed that without departing from the usual strainless configuration of the glucose ring it was impossible to reach a fiber period of 10.6 A. U. from consideration of a flat chain with one or two glucose residues per period. He then found that without any departure from the conditions of strainlessness, this period could be reached if the units were arranged in such a way that the chain possessed a three-fold screw axis, Plate II, Fig. 2. This could be done by rotation of the units about the glucosidic oxygen bonds. Consistent with the normal intermolecular bond distances and angles, there are two possibilities for the configuration of the glucose ring. In addition there would be two more possibilities, depending on whether a right or left-hand spiral is chosen, giving a total of four possibilities for the chain configuration. The projections of the spirals on the

basal planes in the four cases would fit in triangles of sides of 9 - 10 A. U., 7.5 - 8.5 A. U., 7.5 - 8.5 A. U. and 8.5 - 9.5 A. U., respectively.

Kreger stated that a consideration of optical birefringence of the starch grains supported the proposed chain with three glucose residues per period. A study of the density of starch showed that there must be three chains running through the unit cell. It was found impossible to fit three chains with basal projections of triangles of sides either 7.5 - 8.5 A. U. or 9 - 10 A. U. into a unit cell having a basal plane of 15.6 A. U. by 9.0 A. U., which eliminated this provisional unit cell. Kreger thought it was likely that the unit cell must be chosen as a multiple of the one calculated previously.

The ratio of the axes in the basal plane of the provisional orthorhombic unit cell was found to be $\sqrt{3}$: 1, the ratio of the orthorhombic axes in the basal plane of a hexagonal unit cell. Because of the three-fold symmetry of the chains and the hexagonal ratio of the above-mentioned axes, Kreger thought that the unit cell must be hexagonal. In such a cell with $a_o \simeq 18.0$ A. U. (twice the a_o axis of the provisional cell), the spirals could be arranged in such a way that two chains and two half-chains run through a space equal to that of the provisional orthorhombic cell. In this configuration, 18 chains would run through the hexagonal unit cell containing a total of 54 glucose residues. This configuration was consistent with the experimental mass density. Two possibilities exist for the

packing of the chains in accordance with the spacings observed
and consistent with the experimental density of native starch.
These are shown schematically in Plate II, Fig. 3. Kreger sug-
gested that the fact that these different possibilities exist
might account for the different modifications of native starch.

Senti and Witnauer (14) have obtained fiber diagrams of
alkali amylose produced directly on de-acetylation of clamped
filaments of amylose at 25° C. in a 2 per cent potassium hy-
droxide solution in 75 per cent methanol, ethanol, or saturated
butanol. Filaments giving fiber patterns that corresponded to
the "A" structure were obtained by exposing alkali amylose to
high humidity for several days. The fiber identity period was
found to 10.5 A. U. in agreement with the "B" fiber period pre-
viously reported. In saturated water vapor the "A" structure
changed to the "B" structure. How quickly this took place, the
authors did not state. They apparently made no attempt to in-
dex the unit cell of the "A" modification of starch other than
to calculate the fiber period.

It might be of interest to note Senti and Witnauer's work
on the "V" modification of starch. They indexed the fiber pat-
tern of the "V" modification of starch on the basis of an or-
thorhombic unit cell with axes a_o = 9.0 A. U., b_o = 22.7 A. U.,
and c_o = 12.7 A. U.

It is clear from above considerations that, in order to
gain knowledge of structural changes which take place in stal-
ing bread, it is necessary to determine the structural changes

EXPLANATION OF PLATE II

Fig. 1. Model of orthorhombic unit cell of amylose proposed by Rundle et al. showing the D_2^1-P222 space group. The D_2^2-P22$_1$2 space group would differ only in having chains running the same direction in the unit cell translated with respect to each other one-half along the chain axis.

Fig. 2, a. Diagram showing schematic ∝-glucose units linked in threefold screw symmetry with a period of 10.6 A. U. running forward in a constant direction. The chain is shown in a side view.

Fig. 2, b. The chain seen at a small angle with the direction of the chain axis. Dots indicate position of glucosidic oxygen atoms.

Fig. 3. Diagram of the basal plane of the hexagonal unit cell proposed by Kreger showing the two possibilities for the packing of the chains. The dimensions of the provisional orthorhombic unit cell are indicated. Dots indicate positions of the chain axes.
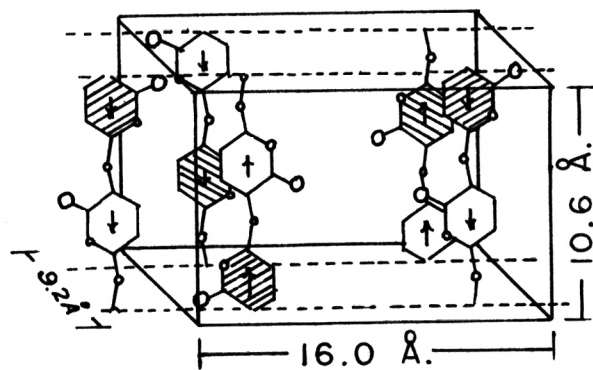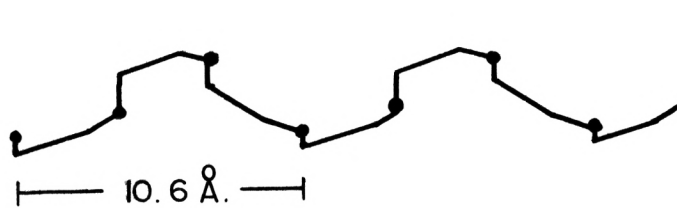
PLATE II

Fig. 1.
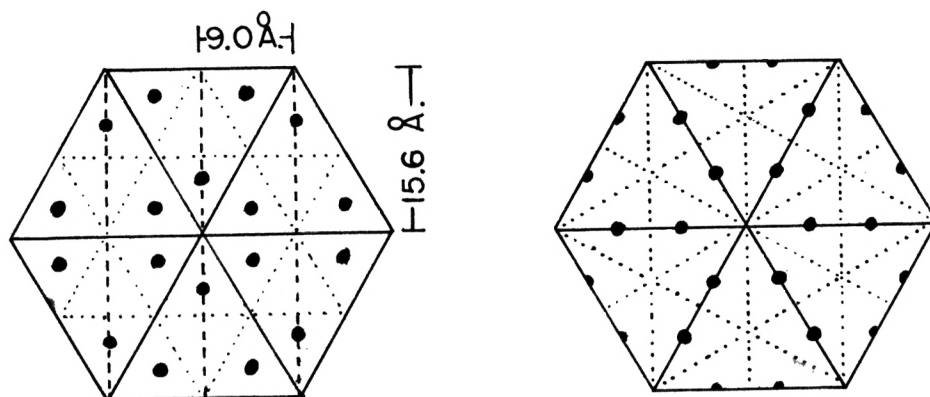

Fig. 2, a.

Fig. 2, b.

Fig. 3.

which take place in the starch contained in the bread. Factors which affect the rate of staling might be expected to affect the structural changes which take place in the starch. The present study is an attempt to observe these structural changes.

APPARATUS

Monochromatic K_α-radiation from the cobalt target of a Machlett x-ray diffraction tube was collimated into a narrow beam by a system of pin-holes. This beam was diffracted from a specimen of bread mounted at the exit port of the collimator onto a flat photographic film approximately two and one-half centimeters behind the specimen. Exposure times were stabilized at approximately two and one-half hours. The specimen holder was rigidly attached to the film holder in order to keep the specimen-to-film distance as nearly constant as possible. Radiation emerged from windows on both sides of the x-ray tube making it possible to obtain diffraction patterns from two specimens simultaneously.

PROCEDURE

Starch crystals in bread are randomly oriented in direction and hence exhibit diffraction patterns which are characteristic of patterns from crystalline powders. A monochromatic x-ray beam passing through such a powder will strike corresponding sets of parallel planes in the individual crystals with

all possible glancing angles and with all possible directions
with respect to a particular crystal axis. Thus, reflection
from a particular set of parallel planes in the crystals as
governed by Bragg's Law will be satisfied by many particles
and the rays, diffracted as cones of radiation, will fall upon
a flat, perpendicular photographic plate as a series of concen-
tric circular rings. Each ring will be uniformly intense
throughout and each will correspond to one set of planes of the
same spacing. Bragg's Law is stated symbolically as,

$$\lambda = 2d \; Sin \; \theta \qquad\qquad -----1$$

where λ denotes the wave-length of the incident radiation, d is
the actual or apparent distance between parallel planes giving
rise to the reflection, and θ is the Bragg or glancing angle.
The radii of these diffraction rings are related to Bragg's Law
by the relation

$$tan \; 2\theta = \frac{S}{R}, \qquad\qquad -----2$$

where θ is again the Bragg angle, S is the radius of the dif-
fraction ring as measured on the film, and R is the specimen-
to-film distance. Thus, by measuring the radii of the rings of
the diffraction pattern, one can calculate the distance between
crystal planes giving rise to the diffraction rings. By assum-
ing the Miller indices of these particular planes and knowing
the distances between the planes, or d-spacings, it is possible
to calculate the dimensions of the unit cell of the crystalline

material.

The optimum specimen thickness for maximum scattering to a spot on the photographic film was calculated by the method below.

The decrease in intensity of an x-ray beam due to absorption in a material is given symbolically by

$$I = I_o \, e^{-\mu t} \qquad \text{-----3}$$

where I is the intensity of the beam after passing through a thickness, t, of the material, I_o is the initial intensity of the beam incident upon the material, and μ is the linear absorption coefficient of the material expressed in centimeters^{-1} units.

As the thickness, t, of the absorbing material increases, the absorption of the x-radiation reduces the amount of radiation transmitted through the material according to equation 3. At the same time, the total amount of radiation scattered to a diffraction spot on the photographic film increases with the cross sectional area of the material that the x-ray beam strikes, or approximately as t^2 (Buerger, 2). This may be expressed in symbols as

$$I_{\text{longest path}} = I_\ell \propto I_o \, e^{-\mu t} \, t^2,$$

or

$$I_\ell = KI_o \, e^{-\mu t} \, t^2, \qquad \text{-----4}$$

where K is a constant of proportionality. This net intensity
which is scattered to a diffraction spot on the film is a maxi-
mum when the first derivative is zero. Setting the first de-
rivative with respect to the thickness, t, equal to zero, gives

$$\frac{dI_\ell}{dt} = KI_o \, e^{-\mu t} \, (2 - \mu t)t = 0. \qquad \text{-----}5$$

It is obvious that the expression can be zero in the non-triv-
ial case only when the expression in the parentheses is zero.
Thus,

$$2 - \mu t = 0$$

and

$$t = \frac{2}{\mu}. \qquad \text{-----}6$$

Equation 6 gives the thickness of the specimen in centi-
meters which will give maximum intensity of scattering for a
particular wave-length of incident radiation. That this inten-
sity is a maximum can easily be shown.

The second derivative of equation 4 with respect to the
thickness, t, is

$$\frac{d^2 I_\ell}{dt^2} = KI_o \, e^{-\mu t} \, (\mu^2 t^2 - 4\mu t + 2). \qquad \text{-----}7$$

Substitution of equation 6 into 7 gives

$$\frac{d^2 I_\ell}{dt^2} = -2KI_o \, e^{-2}. \qquad \text{-----}8$$

The constant K must be positive in order that, according to equation 4, the intensity decrease with thickness due to absorption. Therefore, the negative sign in equation 8 proves that equation 5 gives a maximum value of scattered intensity with $t = \frac{2}{\mu}$.

The linear absorption coefficient, μ, for bread was calculated to be 2.4 centimeters^{-1}. This yields an optimum specimen thickness for bread of

$$t = \frac{2}{2.4} = .85 \text{ centimeter.}$$

Fresh bread baked in the Pilot Bakery of the Department of Flour and Feed Milling Industries, Kansas State College, was employed throughout this study. Slices whose thicknesses were estimated with the eye as being approximately the optimum thickness were cut from the centers of the loaves of bread. It was necessary to compress the bread slices to a thickness of one millimeter or less in order that the specimen thickness be small compared with the specimen-to-film distance. A specimen thickness which was large compared to the specimen-to-film distance would cause the diffraction rings on the film to be considerably broadened thus introducing an appreciable uncertainty in the calculation of the d-spacings. A circular disc, one-quarter inch in diameter, was then cut from the compressed bread slice and mounted in a one-quarter inch diameter circular hole in the brass specimen holder. The specimen was then covered on both sides with sheets of thin Saran plastic and the

plastic was then sealed with Scotch tape in order to minimize
moisture loss from the specimen. A diffraction pattern of the
Saran plastic alone was made in order to eliminate from the
bread patterns any diffraction ring contributed by the plastic.

After irradiating the bread specimen with the x-ray beam,
the diffraction rings on the developed photographic films were
measured with an optical comparator employing a metric scale
with a vernier. End points of the diameters of the diffraction
rings were estimated with the eye and then marked by ink dots.
The distances between ink dots were measured with the compar-
ator and then rubbed out and the procedure repeated four to six
times. In this manner an average of several independent meas-
urements was obtained. The d-spacings of the diffraction rings
were then calculated with the application of equations 1 and 2.

DISCUSSION OF RESULTS

A preliminary study using standard bread baked from com-
mercial unmalted patent wheat flour was performed. It was at
first thought that the crystalline organization which sets up
in staling bread might change in structure with time, and the
preliminary study was undertaken to determine if this was the
case. Thirty loaves of bread were baked from a single batch of
dough and then stored in a temperature and humidity controlled
room. Two sampling procedures were used for the preliminary
study. One of the procedures was to prepare a specimen for one
of the x-ray cameras from one of the freshly baked loaves to be

used for all of the subsequent exposures in that camera. This specimen is hereafter referred to as the permanent specimen. The other sampling procedure consisted of preparing a new specimen for each exposure from an uncut loaf taken from the storage room. These specimens are hereafter referred to as the control specimens. It was thought that after a few days of aging, the permanent specimen would be drier than the control specimen even though sealed with plastic. Thus, study of the effect of moisture loss could be made while the study of crystalline growth in staling bread was being performed.

Simultaneous x-ray diffraction patterns were made of both the permanent and the control specimens every day for 18 days, with the first exposure taken approximately two hours after the bread was removed from the baking oven. The diffraction patterns of the two specimens were compared day by day. No significant differences in either relative intensities or d-spacings of corresponding rings of the diffraction patterns were observed until the fifteenth day of the run. In approximately 15 days of aging, a ring calculated to be due to the 15.8 A. U. spacing of the "B" type starch, termed the 1-ring, began to appear in the diffraction pattern of the control specimens. This ring was not observed in the diffraction patterns of the permanent specimen. It was observed that the permanent sample was considerably drier than the control specimens by this time. From the investigations of the dependence of crystalline reorganization of starch upon the moisture content of the gelatin-

ized starch previously discussed, it was concluded in the present study that the dry condition of the permanent specimen caused the "A" type crystalline organization to appear of which the 15.8 A. U. spacing is not characteristic.

The diffraction patterns taken the same day as the bread was baked showed an amorphous pattern with almost no crystalline organization present. By the second day after baking, diffraction rings began to appear in patterns of both specimens. By approximately the eighth day, maximum crystalline reorganization had apparently been reached in both the permanent specimen and the control specimens as the intensity of the rings did not increase appreciably after this time.

In addition to the 15.8 A. U. spacing ring already discussed, three diffraction rings were observed on the diffraction patterns from both types of specimens. These were identified as the 3, 4 and 5-rings in the nomenclature of Katz. These rings are common to both the "A" and "B" types of starch and are comparable both in relative intensities and d-spacings. The "V" type organization observed by Katz in freshly baked bread was not observed in this study, probably because of the intense background which obscured and masked all of the diffraction patterns. This background presented difficulties in measurements made of the diffraction rings, especially in the early part of the runs. The contrast between diffraction rings and background was less than would ordinarily be desired. For example, the 3-ring which Katz reported as being of medium in-

tensity would have to be termed of weak intensity on the basis
of diffraction patterns obtained in this study due to lack of
contrast between the ring and the intense background.

Day by day comparison of diffraction patterns from the
permanent and control specimens showed no noticeable differ-
ences in either relative intensities or d-spacings of the 3, 4
and 5-rings indicating that moisture content did not affect
crystalline growth as far as these spacings were concerned.
Measurement of diffraction ring radii and calculation of d-spac-
ings proved that the crystalline organization which begins to
appear in two-day old bread does not change in structure as the
staling progresses. Crystalline growth increased until about
the eighth day as evidenced by an increase in intensity of dif-
fraction rings during this period. Maximum crystallinity ap-
peared to have been reached by the eighth or ninth day of stal-
ing.

For reasons given above, it was concluded that for the
studies to follow, using staling retardants in the bread spec-
imens, runs of nine day lengths using initially prepared, per-
manently mounted samples would be sufficient to observe crystal
growth with the exception of the 15.8 A. U. spacing.

It was found during the preliminary exposures that the
specimen-to-film distance of the cameras varied slightly from
exposure to exposure making it necessary to calibrate the cam-
era for every exposure. This was accomplished by spreading a
thin layer of magnesium oxide powder over the bread specimens

and sealing it in with the specimens in the brass holders. The magnesium oxide layer was placed on the side of the specimen nearest the film plane. The d-spacings of magnesium oxide are well known. By measuring the radii of the rings on the diffraction pattern due to the magnesium oxide, and applying equation 2, the specimen-to-film distance for each individual exposure could be calculated. This procedure was done for each of the subsequent exposures.

A study of the effect of crystalline growth during the staling process was next performed on bread treated with different types of staling retardants. The three staling retardants used in the bread loaves were: malted wheat flour; Diastase-33, a fungal enzyme; and Rhozyme D-X, a bacterial enzyme. The latter two are commercial products manufactured by Rohm and Haas Company. The relative amounts of enzymatic activities present in the three treated bread loaves were as follows: malted wheat flour and Diastase-33, 910 SKB units (Sandstedt, et al., 13) per 700 grams of flour and Rhozyme D-X, 30 SKB units per 700 grams of flour. Bread dough containing 700 grams of flour is sufficient to make slightly more than two finished loaves. The active ingredient in all three of these staling retardants is an enzyme named alpha-amylase, which acts upon gelatinized starch by breaking chain linkages (Kerr, 8).

In each of the three runs, each employing one of the above staling retardants, a specimen from a standard, untreated bread loaf such as was used in the preliminary study was used as a

control specimen for comparison purposes. The diffraction pattern of the treated specimen was compared day by day with the diffraction pattern of the control specimen and significant differences were observed for all three types of treated specimens.

Diffraction rings were observed one day after baking in the diffraction patterns of all three types of treated bread specimens, while, as before, the untreated specimen did not develop diffraction rings until the second day. This indicates that the substances which retard staling increase the rate of recrystallization of the starch in the bread. This higher degree of recrystallization was observed day by day throughout the length of the runs. A comparison shows that the degree of recrystallization in the treated and untreated bread specimens arranged in decreasing order is: specimen treated with the bacterial enzyme, greatest degree; specimen treated with malted wheat flour, next greatest; specimen treated with the fungal enzyme, next; and the untreated specimen exhibiting the least amount of recrystallization.

Calculation of d-spacings of the corresponding diffraction rings disclosed another significant difference between treated and untreated bread specimens. In Table 2, it is seen that the d-spacings of the 4 and 5-rings of all three treated bread specimens showed an appreciable increase over the corresponding spacings of the untreated bread specimen. Variations in spacings from day to day are observed but the tendency for a shift

Table 2. Tabulation of observed d-spacings showing comparisons between treated and untreated bread specimens during the indicated staling times.

Staling time in days	Untreated bread — Observed d-spacings in Angstrom units			Bread treated with malted wheat flour — Observed d-spacings in Angstrom units		
	d_3	d_4	d_5	d_3	d_4	d_5
2		5.089 ± ~~.016~~ .039	4.335 ± .016 ~~.009~~		5.089 ± ~~.013~~ .062	4.388 ± .008 ~~.006~~
3		5.012 ± ~~.027~~ .038	4.330 ± .025 ~~.014~~	5.839 ± ~~.026~~ .081	5.130 ± ~~.019~~ .023	4.388 ± .006 .006
4		5.068 ± ~~.006~~ .018	4.356 ± ~~.010~~ .012	6.007 ± ~~.011~~ .087	5.238 ± ~~.012~~ .078	4.429 ± ~~.012~~ .035
5	5.873 ± ~~.016~~ .029	5.054 ± .011 ~~.004~~	4.347 ± .012 ~~.003~~	5.912 ± .015 ~~.008~~	5.141 ± ~~.009~~ .011	4.362 ± ~~.008~~ .032
7		5.020 ± ~~.012~~ .030	4.345 ± ~~.006~~ .009	5.932 ± .014 ~~.012~~	5.115 ± ~~.014~~ .037	4.366 ± ~~.012~~ .028
8	5.930 ± .024 ~~.008~~	5.054 ± .011 ~~.004~~	4.351 ± .015 ~~.007~~	5.908 ± .012 .012	5.198 ± ~~.005~~ .046	4.429 ± ~~.006~~ .035
Average over run	5.902 ± .029	5.050 ± .024	4.344 ± .015	5.920 ± .044	5.152 ± .044	4.394 ± .024

Table 2 (cont.)

Staling time in days	Untreated bread			Bread treated with Rhozyme D-X		
	Observed d-spacings in Angstrom units			Observed d-spacings in Angstrom units		
	d_3	d_4	d_5	d_3	d_4	d_5
2		5.089 ± ~~.016~~ .039	4.335 ± .016 ~~.009~~	5.967 ± ~~.012~~ .061	5.153 ± ~~.013~~ .037	4.420 ± ~~.009~~ .014
3		5.012 ± ~~.027~~ .038	4.330 ± .025 ~~.014~~	5.885 ± ~~.014~~ .031	5.165 ± ~~.007~~ .025	4.418 ± ~~.010~~ .016
4		5.068 ± ~~.006~~ .018	4.356 ± ~~.010~~ .012	5.824 ± ~~.029~~ .092	5.183 ± .010 ~~.007~~	4.403 ± ~~.011~~ .031
5	5.873 ± ~~.016~~ .029	5.054 ± .011 ~~.004~~	4.347 ± .012 ~~.003~~	5.858 ± ~~.017~~ .058	5.124 ± ~~.018~~ .066	4.440 ± .017 ~~.006~~
7		5.020 ± ~~.012~~ .030	4.345 ± ~~.006~~ .009	5.975 ± ~~.009~~ .059	5.238 ± ~~.013~~ .048	4.455 ± ~~.010~~ .021
8	5.930 ± ~~.024~~ .028	5.054 ± .011 ~~.004~~	4.351 ± .007 .007	5.987 ± ~~.006~~ .071	5.280 ± ~~.007~~ .090	4.471 ± ~~.006~~ .037
Average over run	5.902 ± .029	5.050 ± .024	4.344 ± .015	5.916 ± .060	5.190 ± .046	4.434 ± .023

Table 2 (concl.)

Staling time in days	Untreated bread			Bread treated with Diastase-33		
	Observed d-spacings in Angstrom units			Observed d-spacings in Angstrom units		
	d_3	d_4	d_5	d_3	d_4	d_5
3		5.132 ± ~~.030~~ .052	4.407 ± ~~.022~~ .070		5.250 ± ~~.018~~ .066	4.532 ± ~~.013~~ .086
4	5.932 ± .024 ~~.002~~	5.103 ± ~~.016~~ .023	4.360 ± ~~.016~~ .023	5.916 ± ~~.024~~ .031	5.207 ± ~~.017~~ .023	4.458 ± ~~.008~~ .012
5		4.992 ± ~~.017~~ .088	4.324 ± .021 ~~.013~~		5.165 ± .008 .019	4.414 ± ~~.009~~ .032
6	6.020 ± ~~.032~~ .086	5.153 ± ~~.024~~ .073	4.381 ± ~~.010~~ .044	5.967 ± .024 ~~.020~~	5.216 ± .016 .032	4.449 ± .010 ~~.003~~
7		5.046 ± ~~.010~~ .034	4.324 ± ~~.010~~ .013		5.144 ± .010 .040	4.489 ± .008 .043
8	5.850 ± ~~.032~~ .084	5.054 ± .008 .026	4.266 ± ~~.006~~ .071	5.959 ± .023 ~~.012~~	5.144 ± ~~.007~~ .040	4.362 ± ~~.007~~ .084
9		5.080 ± .008 ~~.000~~	4.295 ± ~~.010~~ .042		5.165 ± .008 .019	4.418 ± ~~.007~~ .028
Average over run	5.934 ± .065	5.080 ± .043	4.337 ± .041	5.947 ± .026	5.184 ± .034	4.446 ± .042

in spacings is unmistakable. The average shift in the spacings
of the two rings in all three types is approximately 2 to $2\frac{1}{2}$
per cent. The meaning of the two limits of experimental error
for each d-spacing is explained in Discussion of Errors sec-
tion.

In order to determine the direction of these shifts in in-
terplanar spacings with respect to the axes of the unit cell of
starch, a calculation was carried out to determine the (hkℓ)
indices corresponding to each diffraction ring of the untreated
bread pattern. This calculation was done on the basis of the
hexagonal unit cell for starch proposed by Kreger. The reason
for this choice of unit cell will be discussed later. The re-
sults of this calculation are shown in Table 3.

Table 3. (hkℓ) indices of diffraction rings Nos. 3, 4, and 5.

D-spacing	:	Possible (hkℓ) indices
d_3 = 5.902 A. U.		(210) (3$\bar{1}$0) (3$\bar{2}$0)
d_4 = 5.050 A. U.		(102) (1$\bar{1}$2)
d_5 = 4.344 A. U.		(310) (4$\bar{1}$0)

No generalizations regarding the direction of the shift in
spacings can be drawn from the information above. However, if
d_5 is taken as 4.378 A. U., which is the average value of d_5

taken at its upper limit of uncertainty, then the (hkℓ) indices of this spacing are calculated to be either (202) or (2$\bar{2}$2). It can then be seen that for the unshifted spacing, d_3, the ℓ index, which corresponds to points along the chain or fiber axis of the starch crystal in reciprocal lattice space, is always zero, while in both of the shifted spacings, d_4 and d_5, the ℓ index has a value of two. This could be interpreted as an indication that the shift in spacing of the unit cell of starch in the treated bread is along the chain axis. Since the spacings are increased from 2 to 2$\frac{1}{2}$ per cent, this would indicate that the starch fiber axis has been stretched from 2 to 2$\frac{1}{2}$ per cent, or from 10.6 A. U. to 10.8 or 10.9 A. U. by the action of the alpha-amylase.

DISCUSSION OF ERRORS

The average error, η, of a set of n observations, is the average of the errors of all the observations considered without regard to sign. The probable error, r, is of such magnitude that the error of a single observation is as likely to be within as without the limits of $\pm$r. The relationship between r and η can be shown to be

$$r = 0.8453\eta.$$

The probable error of the arithmetic mean, r', can easily be derived. If a_1, a_2, a_3, $\cdots\cdots$ a_n be n independently observed values, each with a probable error of r, then

$$\overline{a} = \frac{1}{n} (a_1 + a_2 + a_3 + \cdots + a_n)$$

and

$$r'^2 = \frac{1}{n^2} (r^2 + r^2 + r^2 + \cdots \text{ to n terms}).$$

This reduces to

$$r'^2 = \frac{nr^2}{n^2} = \frac{r^2}{n}.$$

The probable error of the arithmetic mean is therefore

$$r' = \frac{r}{\sqrt{n}} = \frac{.8453 \, \eta}{\sqrt{n}}.$$

In Table 2, the first limit of experimental error after each d-spacing represents the probable error of the arithmetic mean, r', for each set of observations. The second limit after each d-spacing represents the deviation of each spacing from the arithmetic mean at the bottom of the column. The two limits for each d-spacing were compared and the smaller limit struck out. The sum of the larger limits was averaged arithmetically to give the average deviation from the arithmetical mean at the bottom of each column.

It is seen that in the d_4 and d_5 spacings, no overlapping of limits of experimental error between the untreated bread and the treated bread occurs for either the Rhozyme D-X or Diastase-33 treatments. There can be observed a slight overlapping of the d_4 spacing at the extreme limits of experimental error between one of the untreated samples and the bread treated with malted wheat flour. A complete overlapping in the case of the

d_3 spacing is observed indicating that this spacing experienced little or no shift under the action of alpha-amylase.

The above would clearly and conclusively indicate a shift in the d_4 and d_5 spacings of starch when acted upon by alpha-amylase.

In addition to the random errors observed from day to day, a constant error was introduced into the calculation of the d-spacings by the method of calibration of the camera. The magnesium oxide layer was placed on the side of the specimen nearest the film plane and, thus, the specimen-to-film distance, R, was measured from the film plane to the face of the specimen nearest the film plane. The thickness of the specimen, as measured with a vernier caliper, was found to be approximately 0.8 millimeters in thickness. The diffracting plane of the specimen can be considered to be at the center of this thickness or approximately 0.4 millimeter from the specimen edge. Thus, the measured value of R was too short by a constant amount of 0.4 millimeter in a total of approximately 22.5 millimeters. The deviation in R was thus approximately

$$\frac{\Delta R}{R} = \frac{0.4}{22.5} = .018$$

or approximately 1.8 per cent. This would mean that all of the calculated d-spacings are too small by the same percentage. This constant error would affect the calculation of the d-spacings but would not affect the amount of shift in the spacings

for the reason that the error is constant and in the same direction.

CONCLUSION

Investigation has shown that the crystalline reorganization of starch which appears in staling bread increases in crystal growth with time up to approximately eight or nine days. Once the crystallinity makes an appearance, it does not change in structure with time. The starch in bread which had been treated with the enzyme alpha-amylase showed changes in both rate of recrystallization and in structure. The starch in the bread which had been acted upon by the enzyme exhibited crystallinity after one day of aging compared to the untreated starch which began to exhibit crystallinity in two days. This increased rate of recrystallization was apparent throughout the staling times studied.

Measurement of d-spacings showed shifts toward larger spacings of the Nos. 4 and 5-rings of the diffraction patterns of the treated bread specimens. The spacing of crystal plane giving rise to the 3-ring apparently was not increased. Calculation of the $(hk\ell)$ indices of the observed rings on the basis of the hexagonal unit cell proposed by Kreger showed that the two diffraction rings which were shifted by enzymatic activity involved indices of the chain or fiber axis of the starch crystal while the unshifted spacing did not involve indices in the direction of the fiber axis. This would seem to indicate that

enzymatic action on the starch chains causes a 2 to $2\frac{1}{2}$ per cent stretching along the chain axis.

It was stated earlier that the (hkℓ) indices of the observed d-spacings were calculated on the basis of the hexagonal unit cell proposed by Kreger. Considerations of optical birefringence of the starch crystals would seem to support this cell rather than the orthorhombic cell proposed by Rundle, et al. Rundle himself stated that in convergent polarized light the crystalline component of whole starch, amylose, in the form of thin stretched films exhibited a uniaxial, optically negative interference pattern. Crystals of high order symmetry such as hexagonal, tetragonal or cubic crystals exhibit uniaxial interference figures, while crystals of lower order symmetry such as orthorhombic, monoclinic or triclinic crystals exhibit biaxial interference patterns (Tutton, 15). For this reason, the orthorhombic cell proposed by Rundle was thought to be unlikely and Kreger's hexagonal cell was used in the calculations of the (hkℓ) indices.

FUTURE STUDIES

It is generally thought that the staling property of bread is a direct consequence of the growth of the recrystallization process in the gelatinized starch in the bread. The present investigation has shown that alpha-amylase, which has the generally accepted property of retarding bread staling, actually increases the rate of recrystallization of the gelatinized

starch. It would appear that a re-examination of the mechanism of bread staling would be warranted in the light of results of the present investigation.

It should be interesting to compare separately the effect of alpha-amylase on the crystalline amylose component of starch with the effect of alpha-amylase on the branched amylopectin component. The alpha-amylase attacks and breaks chemical linkages in both types of starch. This comparison might be made by baking loaves of bread using specially prepared doughs each containing only the one starch fraction. In this way, one might be able to learn something of the mechanism of crystalline growth of the starch in the bread.

In the present investigation, the daily exposures gave little information about the mechanism of crystalline growth. Future studies might include a series of exposures made comparatively often during the first two or three days of aging. Verbal information obtained from Miller and Johnson of the Milling Department of Kansas State College indicated that the staling process is effectively halted when the temperature of the bread is kept a few degrees below zero Centigrade. It might be possible to freeze bread specimens after certain times of staling and thus observe the crystalline growth at frequent intervals.

The x-ray camera equipment could be considerably improved regarding the calibration procedure. As stated earlier, the specimen-to-film distance varied from exposure to exposure mak-

ing it necessary to calibrate each diffraction pattern with magnesium oxide. The specimen holder should be more rigidly attached to the film holder in any future study in order to eliminate this variation in distance, thus making only one initial calibration necessary. Part of this variation could probably be attributed to expansion and contraction of the wooden base of the camera with changes in humidity.

It is readily apparent that the bread staling problem remains an important and as yet unsolved problem. Much future work is needed both on isolated starch systems and on starch systems in the bread itself.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Bear, R. S., and D. French. The significance of x-ray
    diffraction patterns obtained from starch granules.
    J. Am. Chem. Soc., 63 (1941), 2298-2305.

2. Buerger, M. J. X-ray Crystallography. New York: J$_o$hn
    Wiley and Sons, 1942.

3. Clark, G. L. Applied X-rays. New York: McGraw-Hill,
    1940.

4. Hellman, N. N., B. Fairchild and F. R. Senti. The bread
    staling problem. Molecular organization of starch upon
    aging concentrated starch gels at various moisture lev-
    els. Presented at the meeting of the American Associ-
    ation of Cereal Chemists in Denver, Colo., May 24-27,
    1954.

5. Katz, J. R. X-ray investigation of gelatinization and
    retrogradation of starch in its importance for bread re-
    search. Baker's Weekly, 81 (March 24, 1934), 34-37, 46.

6. Katz, J. R., and Th. B. van Itallie. Abhandlungen zur
    physikalischen Chemie der Starke und der Brotbereitung.
    V. Z. fur Physikalische Chemie, A 150 (1930), 90.

7. Katz, J. R., and L. M. Rientsma. Abhandlungen zur phys-
    ikalischen Chemie der Starke und der Brotbereitung. II.
    Z. fur Physikalische Chemie, A 150 (1930), 60.

8. Kerr, R. W. Chemistry and Industry of Starch. New York:
    Academic Press, 1950, 2nd Ed.

9. Kreger, D. R. The configuration and packing of the chain
    molecules of native starch as derived from x-ray dif-
    fraction of part of a single grain. Biochimica et Bio-
    physica Acta, 6 (1951), 406-425.

10. Meyer, K. and G. Pankow. Sur la constitution et la struc-
    ture de la chitine. Helv. Chim. Acta, 18 (1935), 589.

11. Miller, B. S., J. A. Johnson and D. L. Palmer. A compar-
    ison of cereal, fungal and bacterial alpha-amylases as
    supplements for breadmaking. Food Technology, 7, No. 1
    (1953), 38-42.

12. Rundle, R. E., L. Daasch and D. French. The structure of
    the "B" modification of starch from film and fiber dif-
    fraction diagrams. J. Am. Chem. Soc., 66 (1944),
    130-134.

13. Sandstedt, R. M., E. Kneen and M. J. Blish. A standardized Wohlgemuth procedure for alpha-amylase activity. Cereal Chemistry, 16 (1939), 712-723.

14. Senti, F. R. and L. P. Witnauer. Oriented filaments of amylose and alkali amylose. J. Am. Chem. Soc., 68 (1946), 2407.

15. Tutton, A. E. H. Crystalline Form and Constitution. London: The Macmillan Company, 1926.

16. Whistler, R. L. and C. L. Smart. Polysaccharide Chemistry. New York: Academic Press, 1953.

AN X-RAY STUDY OF THE CHANGES IN THE STRUCTURE
OF WHEAT STARCH IN STALING BREADS

by

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X-ray powder patterns of the crystalline starch component
of bread were exposed on photographic films at daily intervals
as the bread staled. It was found that the degree of crystal-
line reorganization, which appears in the starch component of
staling bread, increases with time, apparently reaching a max-
imum in approximately eight or nine days after the bread is
baked. Once the crystallinity appears, it does not change in
structure as the degree of staling develops, but merely in-
creases in crystalline growth.

The effect of the enzyme alpha-amylase, a bread staling
retardant, upon crystalline growth and structure of starch in
staling bread was studied by comparing x-ray powder patterns of
standard, untreated bread with patterns of bread treated with
alpha-amylase enzyme. Diffraction patterns of specimens from
three loaves of bread, each containing a different source of
alpha-amylase, were compared day by day with diffraction pat-
terns of a standard, untreated bread specimen. The three al-
pha-amylase sources were malted wheat flour, fungal Diastase-33
and bacterial Rhozyme D-X.

Comparison of diffraction rings of the x-ray powder pat-
terns showed that all three treated bread specimens exhibited
an increased rate of recrystallization of the starch component
of bread over the rate of recrystallization of the untreated
specimen. This increased rate of recrystallization was ob-
served throughout the staling periods studied.

Measurement of diffraction ring radii and calculation of

d-spacings showed that two of the three diffraction rings studied were shifted in the direction of larger d-spacings in each of the three treated bread specimens by approximately 2 to $2\frac{1}{2}$ per cent over the corresponding d-spacings of the standard, untreated specimens. Calculation of (hk$l$) indices on the basis of the hexagonal unit cell proposed for starch by Kreger indicated that this shift in d-spacings was in the direction of the starch chain or fiber axis. The third, unshifted d-spacing corresponded to (hk$l$) indices not involving the chain axis, indicating further that the shift in d-spacings was along the chain axis. This shift in d-spacings along the chain axis indicated that the starch chain axis was stretched from 2 to $2\frac{1}{2}$ per cent as a result of the action of the alpha-amylase enzyme.

This investigation has shown that the rate of recrystallization of the starch in staling bread is increased by enzymatic action of staling retardants and that this enzymatic action affects the chain linkages in the starch in such a way that the starch chain axis is stretched approximately 2 to $2\frac{1}{2}$ per cent under this action.