THE UNIVERSITY OF MANITOBA

FREE RADICALS IN FLOUR AND DOUGH

by

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ABSTRACT

Ronald Joseph Wasik, B. Sc., The University of Manitoba, February 1971. Free Radicals in Flour and Dough. Major Professor: Dr. W. Bushuk

The detection and identification of the free radicals presumed to be produced in wheat flour-water doughs by mixing was attempted using direct (electron spin resonance - ESR) and indirect (free radical scavengers) techniques. No evidence was obtained to support the hypothesis that free radicals are produced in doughs during mixing.

The production of free radicals by ball-milling flour, gluten and starch and by γ-irradiating flour and gluten was studied using ESR techniques. Both treatments produced free radicals in the respective materials examined. The spectra of ball-milled flour and gluten were similar but different from the spectrum for the starch, indicating that the free radicals in the former materials were different from those in the latter. The concentration of free radicals in ground gluten was much higher than in ground flour or starch. These free radicals were quite stable under vacuum at -196°C but decayed gradually at room temperature to a lower concentration of relatively stable free radicals. In several experiments, free radicals were detectable in ball-milled flour after 90 days storage at room temperature. In attempts to obtain information on their nature, the effects of oxygen, tritium sulfide, sulfur dioxide, water vapor, dibenzylnitrone and 2-methyl-2-nitroso-butanone-3 on these free radicals were investigated. At room temperature, the free radicals reacted rapidly with tritium sulfide, sulfur dioxide and oxygen. They did not react with the other materials under the conditions used. The free radicals produced by γ-irradiation of flour reacted quickly with oxygen but not with water vapor.

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INTRODUCTION

The possibility that free radicals might be involved in the complex reactions that occur in doughs during the breadmaking process has been speculated for some time. These speculations are based primarily on observations of the changes in the physical properties of doughs caused by minute quantities of chemical improvers or by intensive high-speed mixing. Most of the common chemical improvers are oxidizing agents and therefore could initiate reactions involving free radicals. A chain mechanism would then explain how an extremely small amount of chemical improving agent could give rise to an enormous change in the physical properties of dough. On the other hand, intensive high-speed mixing brings about marked changes in the structure of doughs that affect their subsequent behaviour in the breadmaking process. The desirable effects of this mixing, those that improve the color, texture and volume of the bread, are commonly referred to as mechanical dough development. It has been postulated that mechanical development involves reactions of free radicals that are formed by scission of covalent bonds in the flour constituents. This hypothesis has not yet been confirmed or disproved.

The primary objective of the present study was to obtain direct or indirect evidence of the formation of free radicals in dough during mechanical development and in flour during grinding. The secondary objective was the identification of the nature of these free radicals.

After a number of preliminary experiments, it was realized that the direct detection of free radicals in dough would be extremely difficult by standard electron spin resonance (ESR) spectroscopy. Accordingly, it was decided that an initial detailed study of the

nature of free radicals that can be produced in flour by either grinding or γ-irradiation would be extremely useful to subsequent studies on doughs. The major portion of this thesis is therefore based on the experiments with flour. Although the results of the experiments with doughs were inconclusive, they are also included in the thesis.

ELECTRON SPIN RESONANCE SPECTROSCOPY

The presence of free radicals in chemical systems can be directly verified by electron spin resonance (ESR) spectroscopy. Since ESR spectroscopy is a relatively unfamiliar technique to most cereal chemists, a brief discussion of the theory and applications of this technique follows.

Each electron in an atom has a characteristic spin. In a covalent bond, the two electrons involved have opposed or "paired" spins and consequently there is no net magnetic moment resulting from the spin of these electrons. When a molecule with paired spins is placed in a magnetic field, a magnetic moment is induced in the molecule which opposes the applied field. Under this condition the electrons tend to move out of the field. Such molecules are said to be diamagnetic. On the other hand, molecules with unpaired electrons have permanent magnetic moments and tend to become oriented in line with the applied magnetic field. This property is known as paramagnetism. In general, molecules or atoms that have an odd number of electrons have at least one of them unpaired. Such species are commonly known as free radicals. Molecules with two or more unpaired electrons are not very common. Some important examples are oxygen, nitrogen dioxide and nitric oxide. The existence of unpaired electrons or paramagnetism can be readily detected and quantitated by a physical technique called ESR spectroscopy. This technique is also known as electron paramagnetic resonance (EPR) spectroscopy. A brief discussion of the principle of this technique follows.

When a free radical is exposed to a magnetic field, there exists an energy difference with respect to the orientation of the unpaired

electrons, depending on whether the magnetic field resulting from the spin of the odd electron is parallel or antiparallel to the direction of the applied field. When the applied energy is equal to the energy of transition of the electron from antiparallel to parallel, or vice versa, a transition occurs and electromagnetic radiation is absorbed. The energy absorbed per quantum corresponds to the energy of transition.

Figure 1 is a plot of the two permitted energy levels, E_H and E_L, of an electron as a function of the applied magnetic field. Transition occurs at a critical magnetic field strength H_o. The energy difference, E, of the transition of the electron spin is equal to hv where h is Planck's constant and v is the frequency of the absorbed radiation. The wavelength of the microwave energy supplied to the system is usually near 3 cm corresponding to low frequencies and energies. The energy difference, E, is also equal to BgH where B is the Bohr magneton and is equal to the magnetic moment of the electron spin, and g is the gyromagnetic ratio which is the ratio of the magnetic moment to the angular moment of the electron, and reflects the interactions of the electron's magnetic moment with the electron's environment. The value of g is about 2.0023 for a free electron and most free radicals. H is the applied external magnetic field expressed in gauss. Experimentally the magnetic field is increased slowly, and the microwave frequency remains constant. At resonance, microwave energy is absorbed and this absorption of energy is recorded as the derivative curve of the absorption peak by the detecting system. Figure 2 is a schematic illustration of the principle of ESR spectroscopy.

Figure 1

A plot of the two permitted energy levels, E_H and E_L, of an electron as a function of the magnetic field.

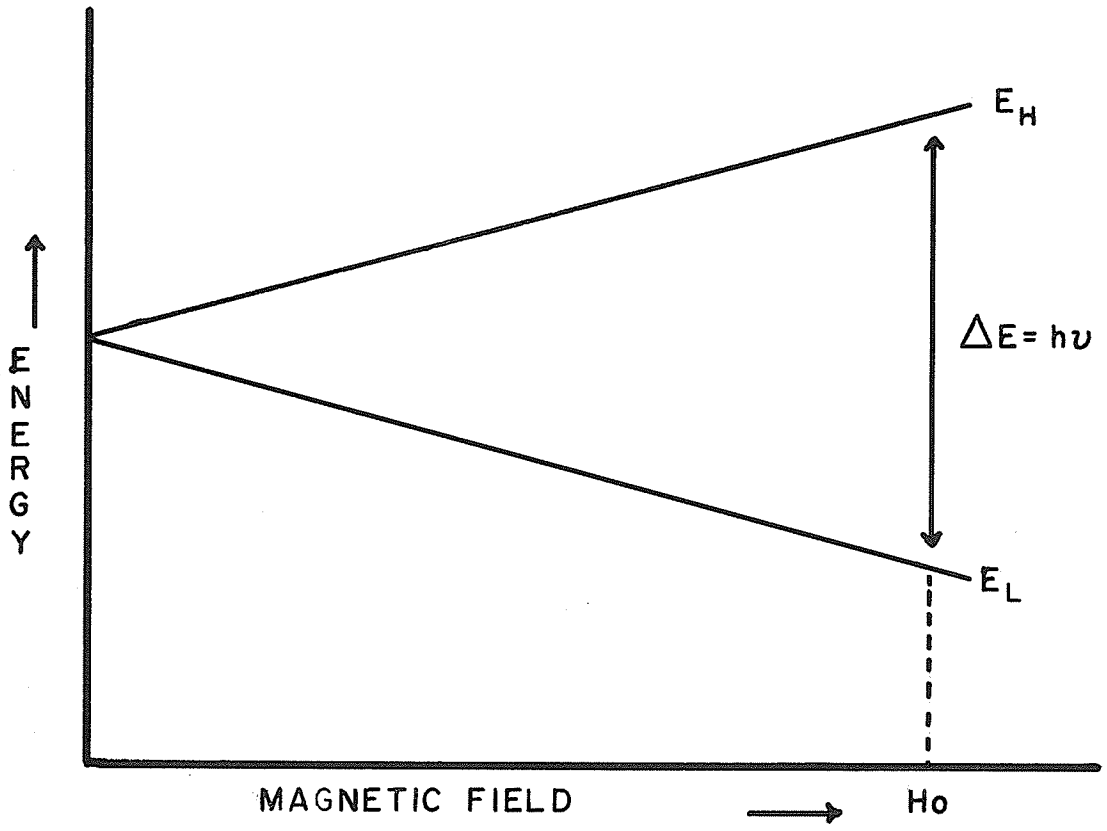

E_H
ΔE = hυ
E_L
ENERGY
MAGNETIC FIELD
Ho

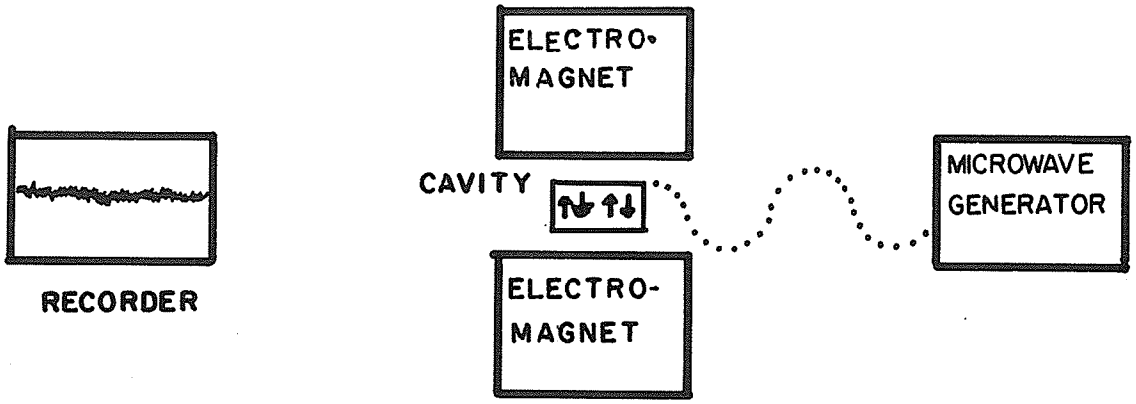
Figure 2

A schematic illustration of the principle of the ESR spectrometer.

(A) Prior to resonance the unpaired electrons in the sample are not oriented.

(B) At resonance the unpaired electrons become oriented.

(A)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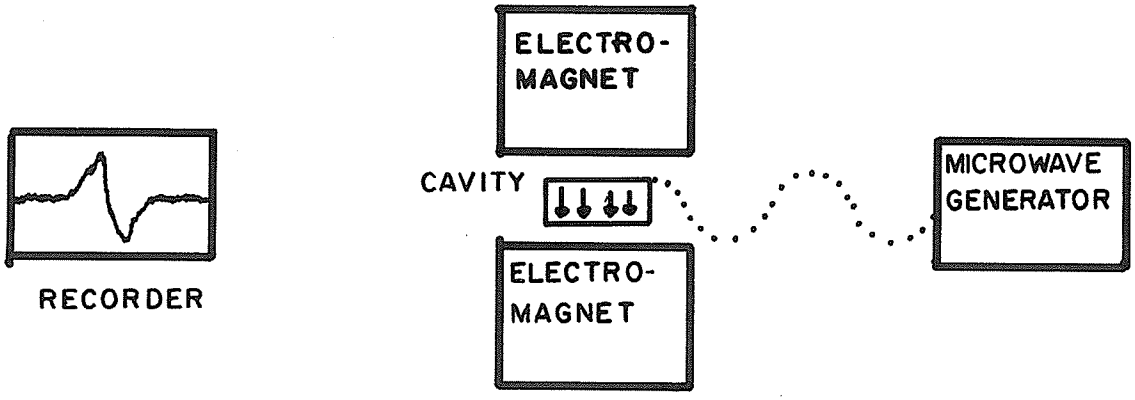
(B)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ESR Signal Analysis

The simplest ESR spectrum consists of a single line as illustrated in figure 3. An ESR line can be characterized by its shape, width, g-factor and intensity. The shape and width are dependent upon relaxation processes which are beyond the scope of this work and therefore will not be discussed.

Figure 4 illustrates the fundamental absorption curve and its first derivative which is actually displayed by the ESR spectrometer. Hpp is the line width between derivative peaks (line width at maximum slope) and Vpp is the peak-to-peak amplitude of the first derivative curve. Vpp is often referred to as the peak height or intensity. Ratios of peak heights and intensities are commonly used to compare ESR spectra. Hpp and Vpp are the parameters used most frequently to compare ESR spectra. Both parameters arise from complicated relaxation processes which are beyond the scope of this work.

The integrated area under the absorption curve is proportional to the spin concentration, and this is equivalent to free radical concentration. Under identical conditions the chart area of the absorption peak may be directly related to the spin concentration independent of the line shape. This requires an absolute standard to calibrate the chart area in terms of concentration of free radicals.

The magnetic field position of an ESR line is given by its g factor ($g = h\nu/\beta H$). For the free electron, $g = 2.0023$. The g-values for most organic radicals are around 2. In other words, the unpaired spin in organic free radicals has very little magnetic moment as characterized by β, the Bohr magneton. Exceptions to this rule occur when the spin is strongly localized as in an oxygen or a sulfur atom.

Figure 3

The simplest ESR spectrum consists of a single line.

H $\longrightarrow$ defines the direction of increasing field strength.

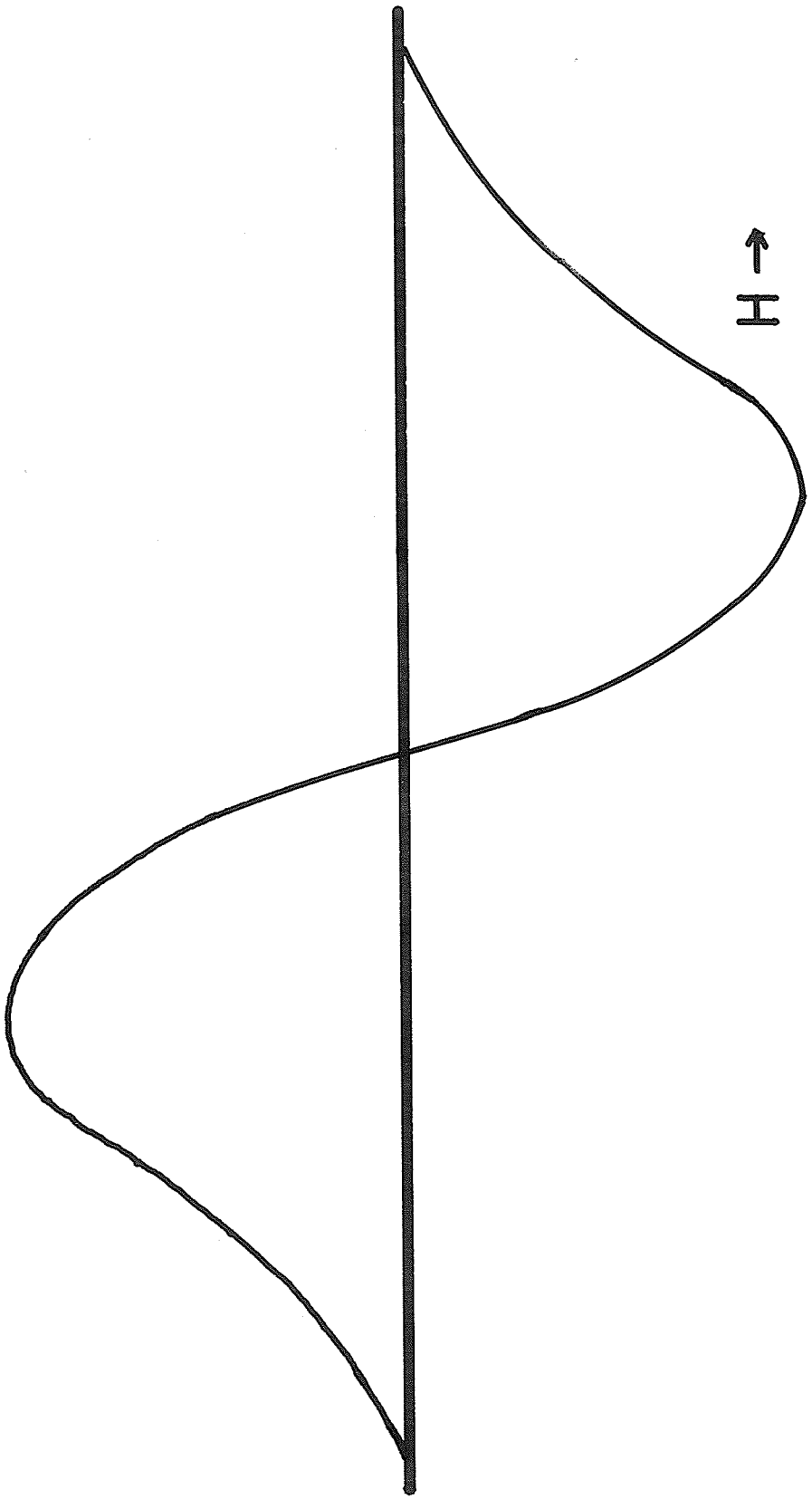
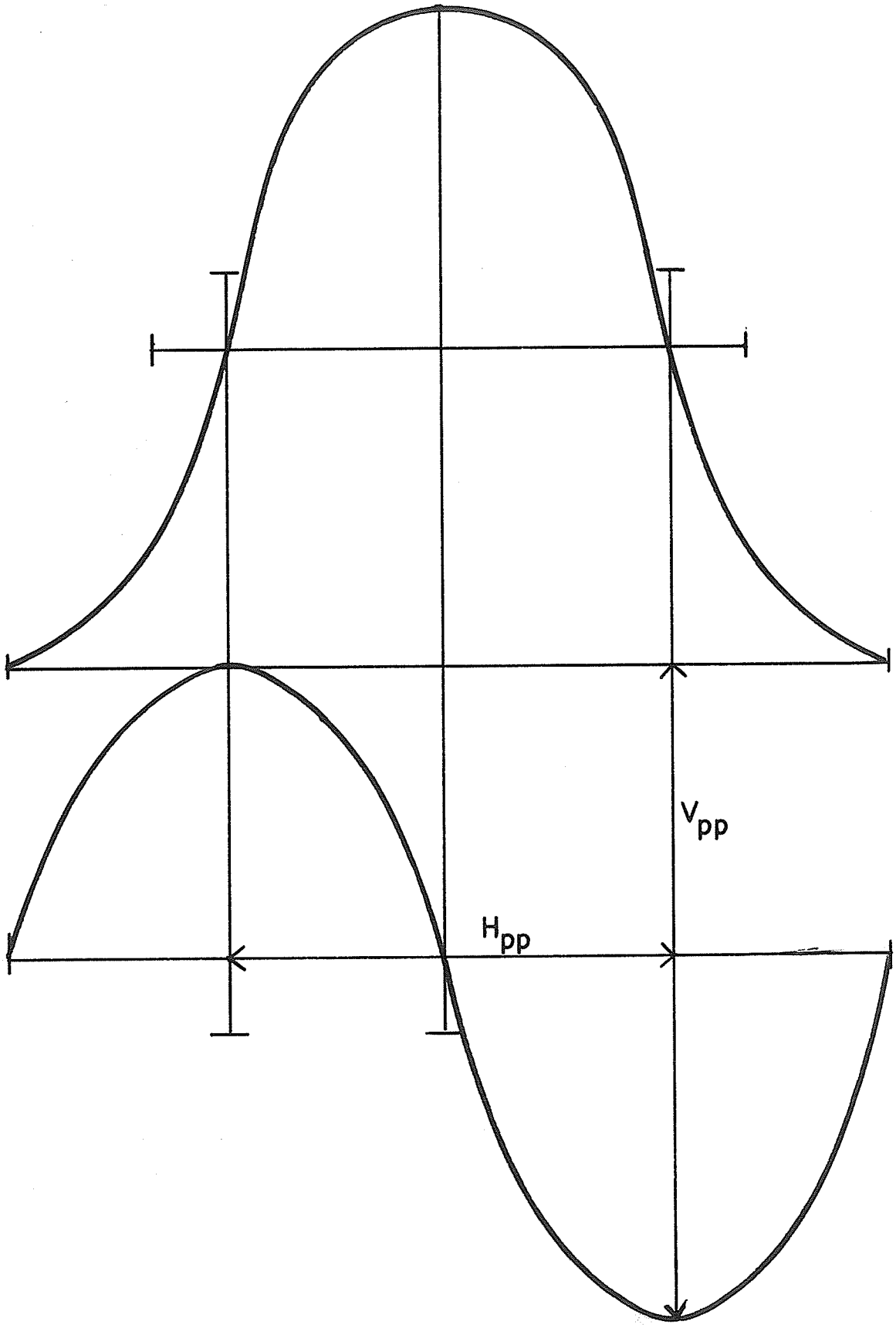

H

Figure 4

This figure illustrates the fundamental absorption curve (bell-shaped) and its first derivative as recorded by the ESR spectrometer.


V_{pp}
H_{pp}

Electron spin may also be affected by the nuclei of other atoms. When this occurs, more energy levels are created and the ESR spectrum becomes complicated with additional lines. This phenomena is referred to as electron-nucleus hyperfine coupling, and is probably the most important interaction for organic free radicals.

ESR in Biology

In biological systems unpaired electrons may exist in two main forms: 1) in free radicals, and 2) in molecules containing transition metal ions in paramagnetic oxidation states. In each paramagnetic centre, the unpaired electron (or electrons) is influenced by the atoms in its immediate environment. In general, ESR provides a tool for detecting and studying paramagnetic sites even if they are present in extremely low concentrations. It is this high sensitivity and specificity which makes the method extremely useful in biological work.

As in other forms of spectroscopy, much information may be obtained by simple inspection of an ESR spectrum. On a superficial level, the technique may be used qualitatively to decide simply if free radicals or transition metal ions are present in the sample. On a more advanced level, the paramagnetic species can be identified and its concentration determined. It is also possible to determine if the species occurs at more than one site, and to obtain information about the symmetry of the bonding at such sites. However, the true value of this method is indicated in its application to the study of the interactions between unpaired electrons and its neighbouring atoms. Finally, in certain cases, ESR studies using fast scanning

techniques may be applied to kinetics of reactions involving free radical intermediates. Such intermediates have been postulated and found in many biological redox reactions.

REVIEW OF THE LITERATURE

Free Radicals Produced by Mechanical Degradation of Macromolecules

Many synthetic polymers (1, 2, 3, 4), cellulose (5, 6), wool (7) and other keratinaceous materials (8) can be readily degraded by mechanical forces. The first step of the degradation process involves the scission of covalent bonds with the formation of free radicals. In relation to these observations, Butjagin and Abagjan (9, 10) ball-milled several proteins at low temperatures and succeeded in producing free radicals that could be detected by standard ESR techniques.

In 1966 Redman et al. (11, 12) extended the work of the Russian workers (9, 10) to include flour, gluten, gliadin and wheat starch. They (11, 12) found that free radicals can be readily produced in these materials by grinding at low temperatures in a vacuum. Free radicals could not be produced by grinding in air. These workers did not identify the sites or the nature of the free radicals. They reported that the radicals were stable at low temperatures but decayed rapidly at room temperature, or when oxygen was admitted to the system. It was postulated that the free radicals in gluten (or flour) resulted from the removal of a hydrogen atom from glycine.

Free Radicals Produced in γ-Irradiation of Flour

In 1962, Lee et al. (13, 14, 15) of the University of Saskatoon, subjected flour, starch and gluten of different moisture content to various doses of γ-irradiation at room temperature. They found that free radicals could be readily produced in flour if the moisture contents were below 8%. No free radicals were detected in flours with higher moisture contents. The disappearance of the free radicals

in flour followed second-order kinetics. The rate was three times faster in flour of 3% moisture than in oven-dried flour. Since the only variable component was water, it appeared that water was reacting directly with the free radicals to yield some non-paramagnetic product. The proposed mechanism for the reaction of the free radicals with water was:

1. $R^\circ + H_2O \longrightarrow RH + OH^\circ$

2. $R^\circ + OH^\circ \longrightarrow ROH$

where

R° = free radical species in flour, and

OH° = hydroxyl radical

Free Radicals Produced in the Dough System

It has long been speculated by cereal chemists that free radicals might be formed during mixing of wheat flour doughs. However, there have been only few attempts to verify or discredit the hypothesis.

In 1968, Dronzek and Bushuk (16) obtained some indirect evidence supporting the formation of free radicals in a dough by mixing. It was found that ^{14}C-methylmethacrylate, which was present in the dough during mixing, polymerized on some flour components since part of the radioactivity could not be removed by solvents in which the methacrylate monomer is soluble. If it is assumed that methylmethacrylate polymerization in dough can be initiated only by free radicals, then the results obtained by these workers indicate that free radicals are produced in dough by mixing. However, the possibility that the monomer reacted with some flour component by a mechanism not involving free radicals has not been completely ruled out.

MATERIALS AND INSTRUMENTATION

The flour used in this study was milled from Manitou, a high-quality hard red spring wheat variety. Its protein and ash contents were 12.5 and .435% respectively on a 14% moisture basis.

Samples of starch and gluten with the exception of the industrial gluten used in our experiments were prepared in our laboratories from Manitou wheat flours. The industrial gluten was manufactured and supplied by Industrial Grain Products Company Limited.*

Because a large variety of other chemicals and reagents were employed throughout the research, they will not be listed, but will appear in the pertinent sections. Standard laboratory reagent-grade chemicals were used.

A standard high vacuum line was used to outgas and introduce desired gases into the sample bulb. The system was capable of achieving and maintaining vacuums of 10^{-3} mm of mercury.

The ESR spectrometer used was a Varian model E-3. The instrument was equipped with a multipurpose cavity.

The grinding was done with a modified Wig-L-Bug dental amalgamator. The arms of the amalgamator were lengthened to permit the immersion of the sample into liquid nitrogen.

A Brabender Farinograph equipped with a 50 gram stainless steel mixing bowl was used to prepare most of the doughs used in this investigation.

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*Thunder Bay, Ontario, Canada

METHODS

Because of the large variety of the experiments that were done only a brief general description of the methods used can be given. Specific details pertaining to individual experiments will be noted in the section dealing with these experiments.

In a typical experiment, flour was introduced into a specially fabricated pyrex bulb containing several pyrex beads. The sample was then outgassed at room temperature on a high vacuum line to 10^{-3} mm of mercury. The sample bulb was sealed and removed from the vacuum line. Figure 5 shows a schematic of the sample bulb.

The grinding was carried out by shaking the bulbs containing the flour and the glass beads with a Wig-L-Bug amalgamator. This arrangement gives a grinding action similar to that of a ball mill. Samples were shaken for about 1.5 hours at 105 volts. For grinding at liquid nitrogen temperatures, the glass bulb containing the flour sample was completely immersed in liquid nitrogen.

On completion of the grinding the sample bulb was removed from the shaker while kept immersed in liquid nitrogen, if necessary. Some of the ground flour was transferred into the pyrex finger which is an integral part of the same bulb. This operation can also be done in liquid nitrogen if required. ESR spectra were recorded at room or at liquid nitrogen temperatures. No special procedure was required to obtain a spectra at room temperature; however, to study the samples at lower temperatures it was necessary to place the sample finger into a special quartz dewar. Figure 6 is a schematic illustration of the arrangement used at room and at liquid nitrogen temperatures.

A Cobalt-60 gamma cell was used as the source of γ-irradiation.

Figure 5

A schematic illustration of the pyrex sample bulb filled with glass balls and a sample to be ground. Scale 1:1

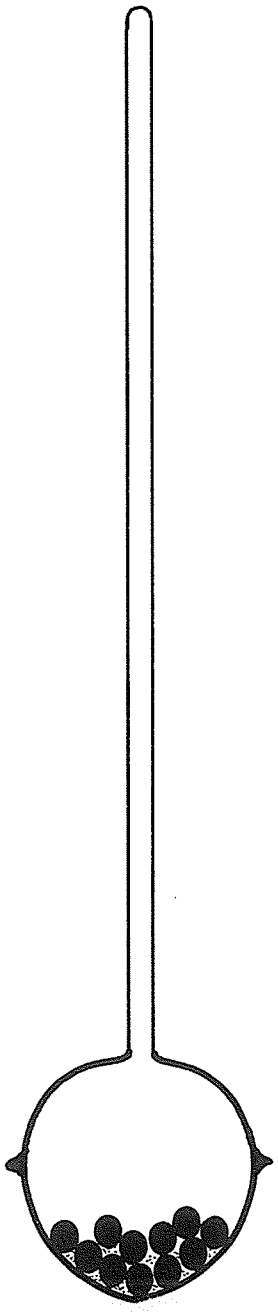
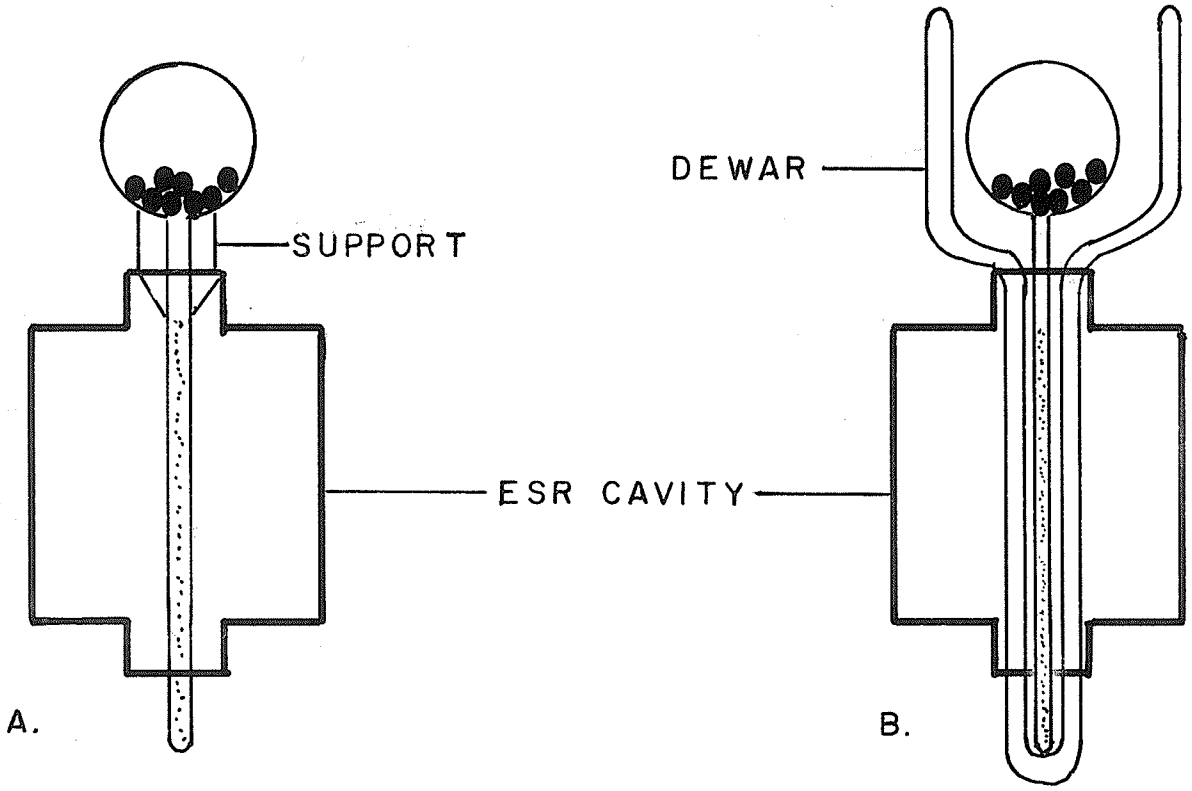


Figure 6

A schematic illustration of the cavity equipment and arrangement employed on the ESR spectrometer to study free radicals at either room or liquid nitrogen temperature.

(A) At room temperature

(B) At liquid nitrogen temperature


SUPPORT
ESR CAVITY
A.
DEWAR
B.

Irradiations at the required exposures were made at room temperature and at liquid nitrogen temperature. In the latter case the sample was held in a pyrex dewar filled with liquid nitrogen which was irradiated together with the sample. The ESR spectra of the irradiated material were recorded in the usual manner.

All grindings and irradiations were made under specified atmospheres, depending on the type of experiment. Actual atmospheres used will be indicated later.

A variety of techniques was used to prepare doughs used. These techniques are described in the appropriate sections.

RESULTS AND DISCUSSION

Free Radicals in Dough

ESR Studies in Doughs Mixed in Air, Nitrogen, and Argon

Although the previous attempts in our laboratory to detect free radicals by ESR spectrometry gave negative results, the experiments were repeated using a more sensitive spectrometer.

For the present experiments, doughs were mixed in air, nitrogen and argon in the farinograph mixer using speeds of 63 rpm. Prior to mixing the doughs in nitrogen and argon, the flour samples and the distilled water were exhaustively outgassed to remove all traces of oxygen.

As quickly as possible after mixing, the dough samples used for ESR examination were drawn into quartz sample tubes by suction. The portion of the tube containing the dough sample was broken off and inserted into a larger quartz tube which was then sealed. The samples were either frozen in liquid nitrogen and examined at -196°C, or examined at room temperature. Due to the high dielectric constant of the water present in the dough samples, it was found that the samples could be monitored properly by the ESR spectrometer only at -196°C and not at room temperature.

None of the six doughs (three different atmospheres and two mixing speeds) showed any ESR evidence of free radicals. Accordingly, if free radicals are produced in doughs during mixing they are scavenged before they can be detected.

Application of 2-Methyl-2-nitroso-butanone-3 and Dibenzylnitrone

Since the attempts to obtain direct evidence of free radicals in dough were unsuccessful, two indirect experimental approaches were

investigated. The first was to use scavengers that are known to react with free radicals in aqueous solution to give a stable free radical. The second approach was similar to that used by Dronzek and Bushuk (16) in which monomers that polymerize by free radical initiation were added to the dough during mixing.

The two free radical scavengers used were 2-methyl-2-nitroso-butanone-3 and dibenzylnitrone. These scavengers gave negative results when used with dry flour (see below). In the present experiments, the scavengers were added to the flour prior to the addition of water. All doughs were mixed in air and in nitrogen at several speeds on the farinograph mixer. None of the doughs examined showed any evidence of free radicals.

Detection of Free Radicals by Graft Polymerization

Certain organic monomers will polymerize in the presence of free radicals. If the initiating free radical is part of a macromolecule, then the polymerization leads to a grafting of a polymer branch onto the macromolecule. If the portion of the graft could be identified, then the identity of the free radical would be determined. This technique was used for grafting polymethylacrylate onto gluten by Wall (17) by producing free radicals on the gluten with sodium hydride using dimethyl-sulfoxide (DMSO) as a solvent. Radioactive methacrylate monomer was used by Dronzek and Bushuk (16) in an attempt to apply this indirect approach to the detection of free radicals in mixed doughs.

In the present study, three types of monomers were used: styrene, methylmethacrylate and hydroxyethylmethacrylate. The monomers were added to the flour (7 ml. monomer added to 50 g. flour) before the addition of water. The doughs were mixed in the farinograph at normal (63 rpm) and at high (160 rpm) speeds.

For analysis, 40 mg. of dough was taken and hydrolysed using the standard procedure for preparing the hydrolysates for amino acid analysis (18). Amino acid analyses were made on a Beckman 121 amino acid analyser. The selection of this analytical technique is based on the assumption that the addition of monomer(s) to a particular amino acid would decrease the content of that amino acid. This procedure would give no information if the grafting sites were in flour components other than proteins.

The results of the amino acid analyses that were obtained are shown in Table 1. Two types of effects were obtained. Firstly, the amounts of some amino acids were significantly lower for doughs containing the monomers compared with the control. These acids were underlined for easy reference. Secondly, there were four unknown components in the hydrolysate of the hydroxyethylmethacrylate treated dough. The nature of these components was not determined.

The dough treated with styrene showed a decrease in the basic amino acids and tyrosine. Methylmethacrylate produced a decrease in all amino acids except cysteine and tyrosine whereas hydroxyethyl-methacrylate had no effect. Because of a malfunction in the amino acid analyser it was not possible to repeat these experiments. They are reported here to indicate that the approach might warrant further study. Pending replication of the data (Table 1) no significance can be attached to the differences that were observed.

Table 1

Amino acid compositions of normal dough and doughs mixed in the presence of styrene, hydroxyethylmethacrylate and methylmethacrylate. The values given are integrator areas for the respective peaks. The letters n.c. mean that the integrator counted no area for that particular elution time, therefore n.c. is equivalent to 0 area.

TABLE 1

Amino Acid	Control	Styrene	Hydroxyethyl-methacrylate	Methyl-methacrylate
lysine	443	147	465	241
histidine	513	146	430	224
arginine	524	165	566	322
unknown	n.c.	n.c.	45	n.c.
aspartic acid	956	906	848	416
threonine	645	637	622	334
serine	1268	1288	1249	690
unknown	n.c.	n.c.	203	n.c.
glutamic acid	7708	7613	6869	3912
proline	798	766	699	383
glycine	1485	1428	1405	727
alanine	1036	973	924	493
cysteine	55	57	n.c.	46
valine	1087	1080	991	515
unknown	n.c.	n.c.	87	n.c.
methionine	200	235	210	155
iso-leucine	921	856	851	418
leucine	1749	1616	1618	847
tyrosine	235	22	288	194
phenylalanine	943	875	1025	537

FREE RADICALS IN FLOUR

Free Radicals Produced in Flour by Grinding

Experiments were carried out under a variety of conditions to gain additional information about the stability and reactivity of free radicals produced by ball-milling flour.

Flour which was ground in a vacuum for 1.5 hours at -196°C gave a strong ESR signal indicating the presence of free radicals (Fig. 7). This spectrum is readily reproducible. A control flour, not subjected to the grinding action, gave no ESR signal. Experiments in which flour was replaced with powdered quartz showed that fragmented pyrex beads were not responsible for the ESR signal. The ESR spectrum shows a triplet. The ratio of intensities of the three peaks was 1:2:1. These spectra were similar to but better resolved than those reported by Redman *et al*. (11, 12) for ball-milled flour.

When the ground sample was allowed to warm up to room temperature there was a large initial loss of the original signal intensity but the remaining free radicals were very stable. Figure 8 illustrates the stabilized spectrum recorded at room temperature at a gain setting four times the gain used for Figure 7 (recorded at -196°C). The overall appearance of the spectrum changed as well. The spectrum was transformed into a doublet with some resolved fine structure at the extreme ends of the spectrum. The ratio of peak intensities was 1:1.

From the results of Figs. 7 and 8, it appears that two types of free radicals are formed in flour by grinding at liquid nitrogen temperatures: 1) those that disappear quickly on warming to room temperature; and 2) those which persist at room temperature. Another possible explanation of these results is that the primary free radicals

Figure 7

The ESR spectrum (recorded at -196°C) of the free radicals produced by ball-milling flour at -196°C for 1.5 hours in a vacuum.

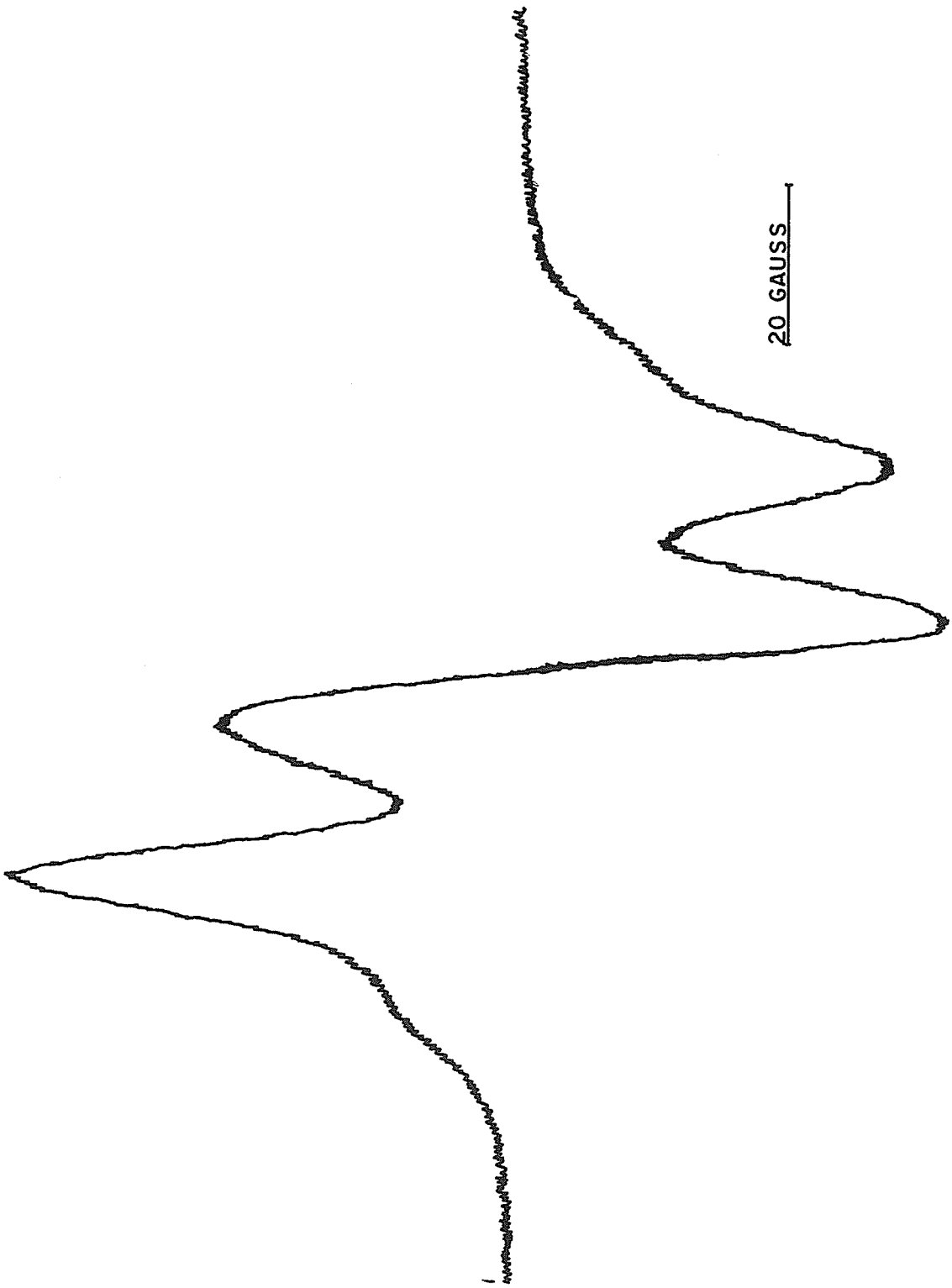
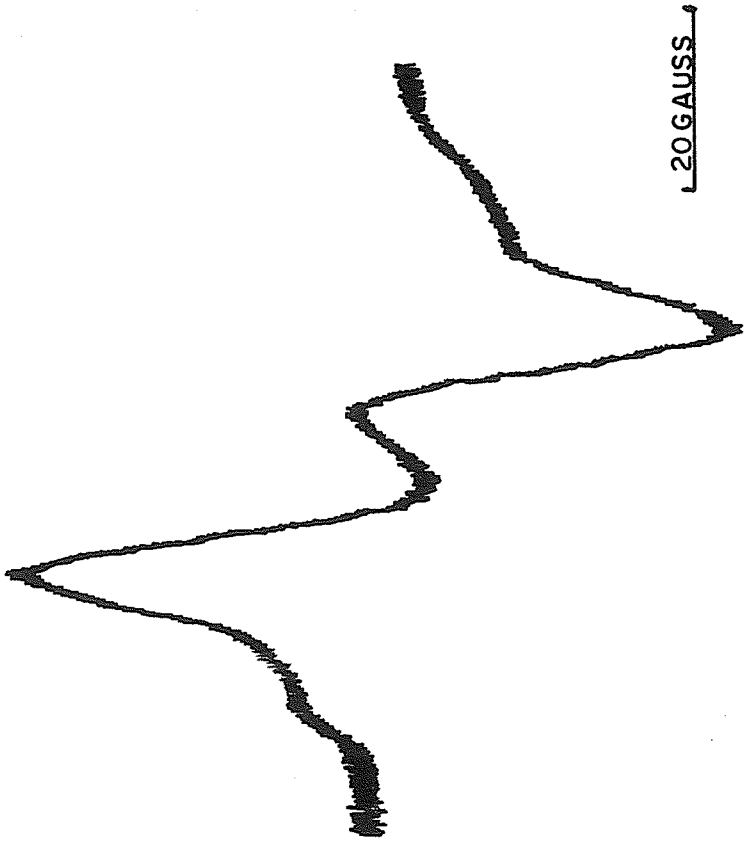

20 GAUSS

Figure 8

The ESR spectrum (recorded at room temperature) of a flour sample which had previously been outgassed and ball milled at -196°C for 1.5 hours.


20 GAUSS

produced by grinding at low temperatures either recombine or react at higher temperatures to form more stable secondary free radicals. The marked decrease in the free radical concentration on warming is more consistent with the recombination mechanism.

A sample of outgassed flour was then milled at room temperature instead of -196°C for the same length of time as in the previous experiment. The resulting spectrum was almost identical with that obtained for the sample ground at low temperature but examined at room temperature (Fig. 9). Presumably in the sample ground at room temperature the highly unstable free radicals disappear quickly after they are formed. The concentration of free radicals produced by grinding at room temperature was slightly higher than the concentration of the free radicals in the sample which was ground at -196°C. It is speculated that this might be due to the fact that proteins are more easily denatured at ambient temperatures than at low temperatures. It would appear that at room temperatures the controlling factor in ground flour is the reaction of free radicals to yield more stable secondary free radicals.

Free radicals were also detected in the flour sample that was milled at -196°C in the presence of air (Fig. 10). This signal faded quickly on warming to room temperature.

No free radicals could be detected when the flour was ground at room temperature in the presence of air (Fig. 11). Presumably the free radicals formed under these conditions disappear quickly by reacting with oxygen.

Figure 9

The ESR spectrum (room temperature) of the free radicals produced by grinding outgassed flour at room temperature for 1.5 hours.

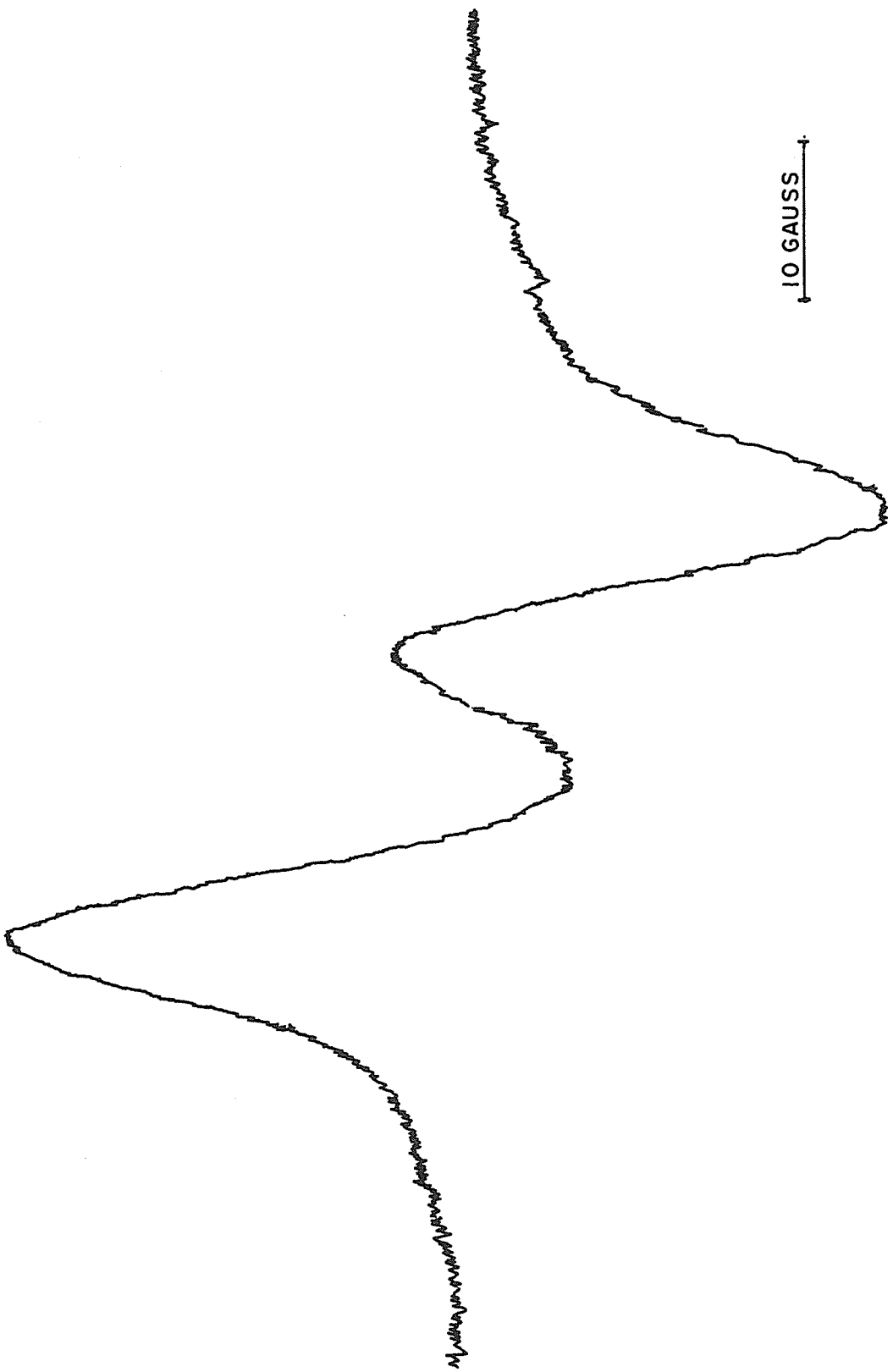

10 GAUSS

Figure 10

The ESR spectrum (recorded at -196°C) of the free radicals produced by grinding non-outgassed flour for 1.5 hours at -196°C.

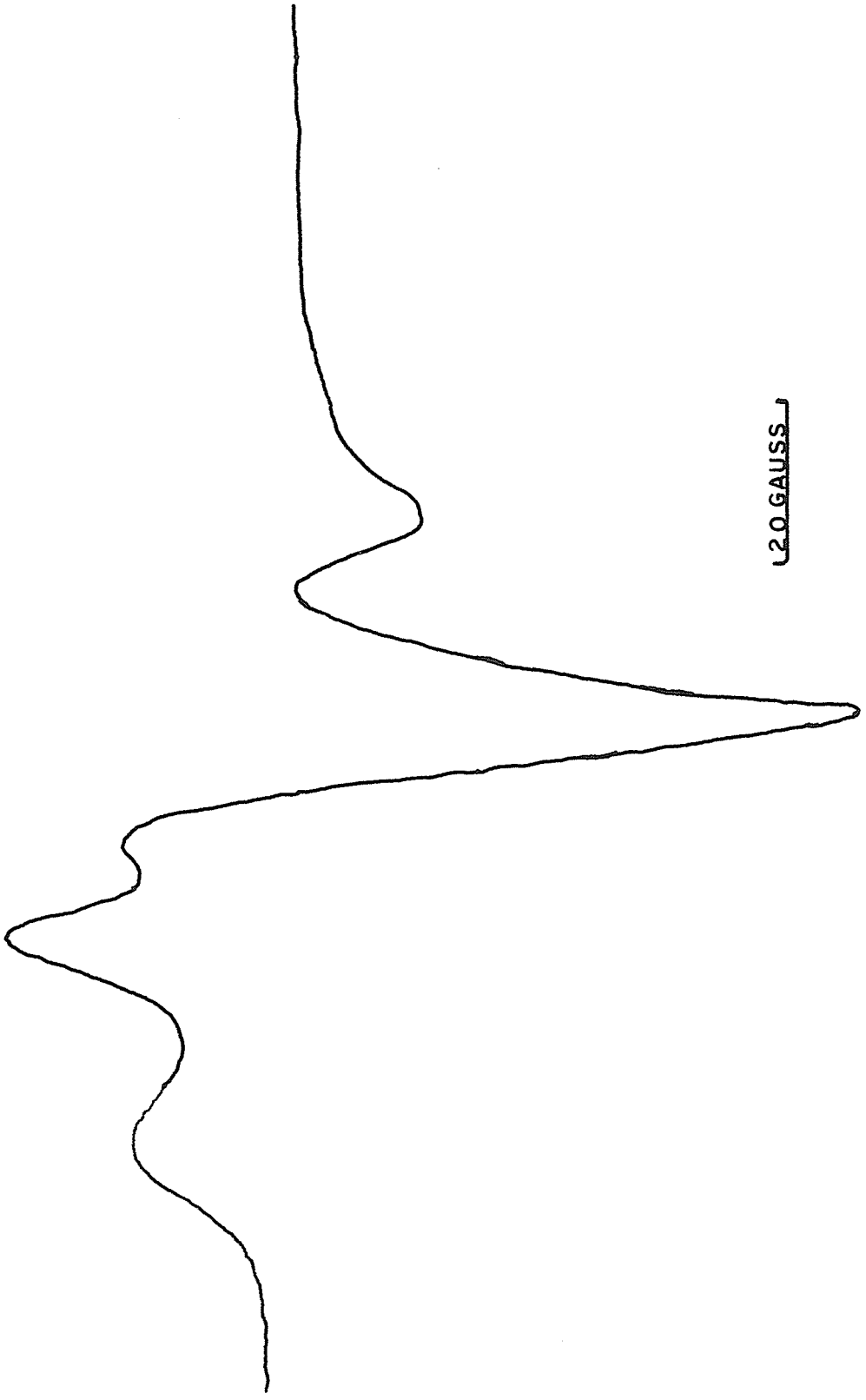

2.0 GAUSS

Figure 11

No free radicals could be detected when flour was ground at room temperature in the presence of air.

Free Radicals in Wheat Starch and Gluten Produced by Grinding

In an effort to identify the nature and site of the free radicals produced in flour by grinding, the two major components of flour, starch and gluten, were ground and examined under the same conditions as flour. Wheat starch ground at -196°C in a/vacuum and subsequently warmed to room temperature showed a somewhat different ESR spectrum from that obtained for flour subjected to identical treatment (compare Fig. 7 and 12). The main difference was qualitative rather than quantitative. The spectrum of starch (determined at room temperature) was a broad triplet with peak intensities of the ratio of 5:1:4.

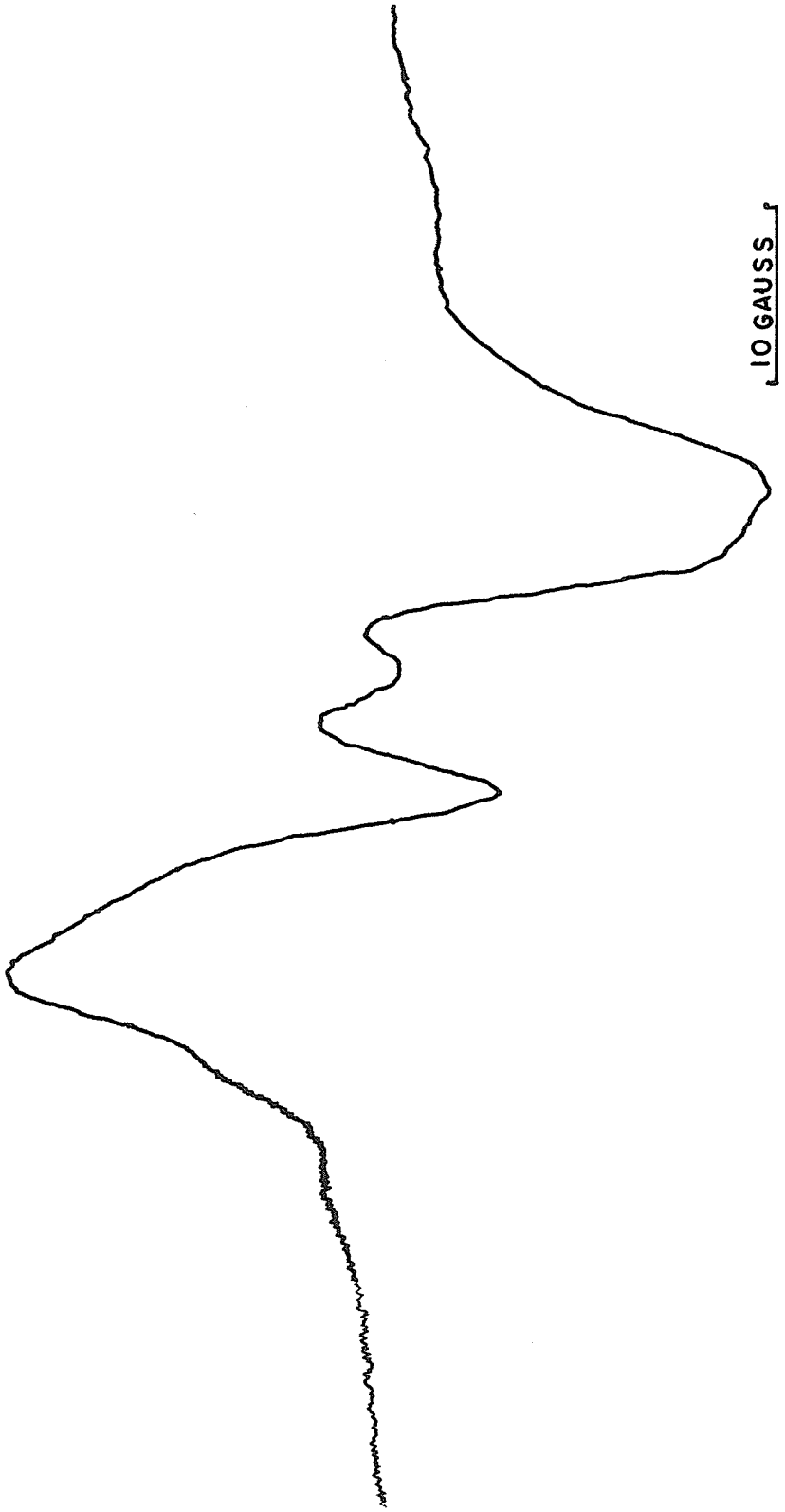
Due to technical problems encountered in building and repairing a large liquid nitrogen dewar used to keep the sample cold while the ESR spectra were being recorded, it was not always possible to make ESR measurements at -196°C. Accordingly in most of the experiments the samples were ground at -196°C but the ESR spectra were determined at room temperature.

Two preparations of dry gluten were used to examine the production of free radicals in this component of flour by grinding. The first was a commercial vital gluten sample provided by Industrial Grain Products and the second was a sample prepared in the laboratory from a flour similar to that used in this study.

The original commercial gluten showed a weak ESR signal prior to grinding. This spectrum was a singlet with no fine structure. The origin and nature of the free radicals that produced this signal were not investigated. On grinding at -196°C and warming to room temperature, this original signal disappeared and a new signal at a lower magnetic field appeared. Fig. 13 illustrates the spectrum of commercial gluten before (A) and after grinding (B).

Figure 12

The ESR spectrum (room temperature) of the free radicals produced by grinding an outgassed starch sample at -196°C for 1.5 hours.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The spectrum of ground commercial gluten consisted of a doublet with a peak intensity ratio of 1:1. The same hyperfine structure was observed.

Samples of gluten prepared in the laboratory had no detectable free radical concentration. After grinding the samples in a vacuum at -196°C and then warming them to room temperature, the spectrum obtained from the resulting free radicals was 20 times more intense than the spectra obtained from radicals produced by milling commercial gluten under the same conditions (compare Fig. 13B and 14). Note the difference in gains used. The peak intensity ratio and overall appearance of these free radicals on the ESR spectrum were very similar to those obtained for ball-milled flour.

These results suggest that the protein rather than the starch component of flour is the major source of free radicals when flour is ground by ball-milling. It has been seen in the case of the prepared gluten that the free radical concentration was much higher than the concentration obtained from ground starch. In the two experiments with gluten the resulting ESR spectra resembled that of ball-milled flour more closely than the spectrum for ball-milled starch.

These results pose an interesting question. Why should proteins which form only about 12% of the flour be the major source of free radicals while starches which form 70-80% of the flour appear to be only a minor source of free radicals? The answer could be in the physical structure of the flour particles. It is known that in flour from hard wheats the starch granules are completely covered with layers of protein (37). If this is so, then the protein would be more subject to the abrasive action during ball-milling than the starch. This hypothesis remains to be verified.

Figure 13A

The ESR spectrum (room temperature) of commercial gluten prior to ball-milling. Gain 5×10^6.

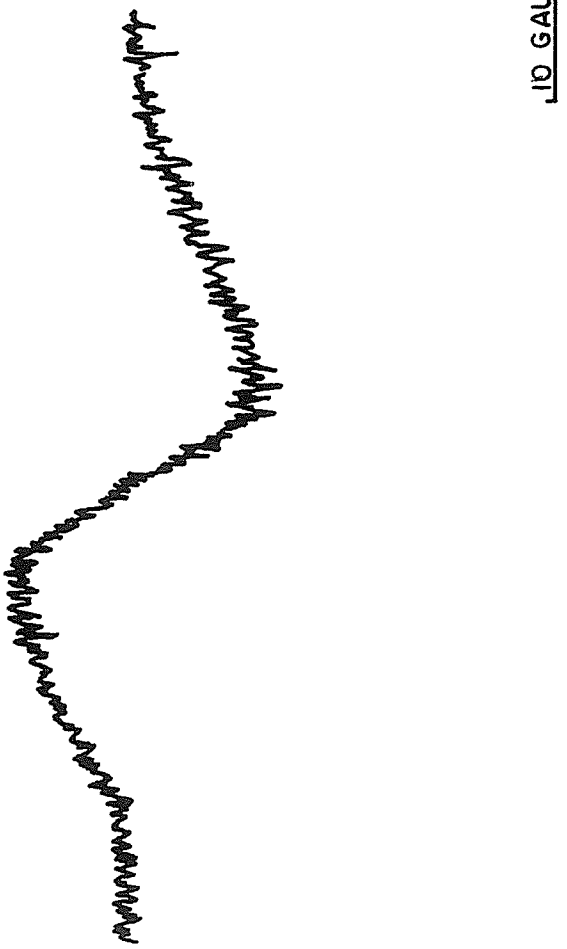
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Figure 13B

The ESR spectrum (room temperature) of the free radicals produced by grinding outgassed commercial gluten for 1.5 hours at -196°C. Gain 10 x 10^5.

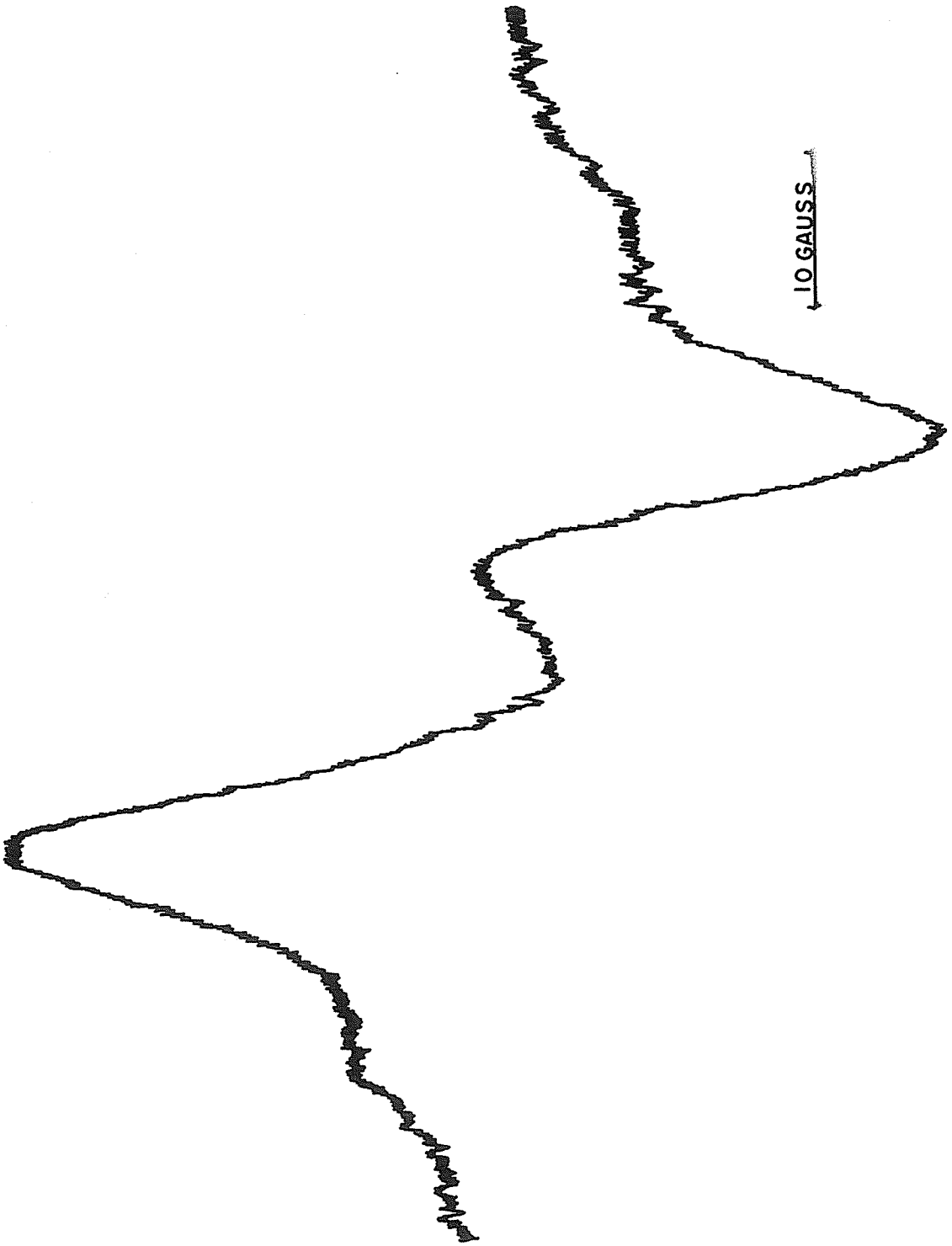
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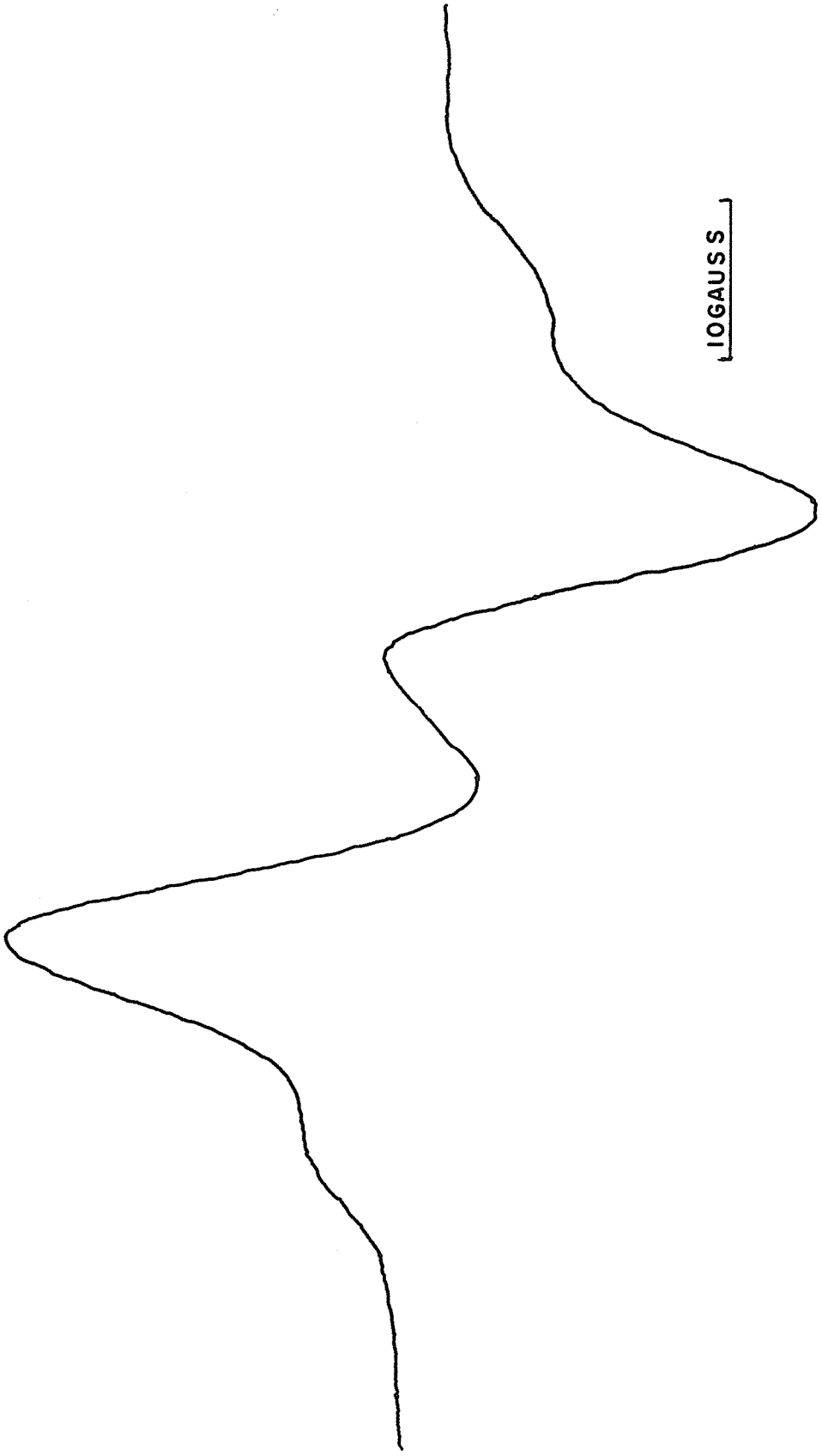
Figure 14

The ESR spectrum (room temperature) of the free radicals produced by grinding the prepared gluten in a vacuum for 1.5 hours at -196°C. Gain 5 x 10^4.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Free Radicals Produced in Flour by Electric Discharges

High voltage electrical discharges are known to produce free radicals in proteins (19, 20). Furthermore, it has been shown that these free radicals result from the abstraction of hydrogen atoms. It was of interest to compare the spectrum of flour subjected to electric discharge with that of flour containing free radicals produced by grinding. Such a comparison might be useful in determining the nature of the free radicals in ground flour.

Figure 15 shows the ESR spectrum of flour exposed to an electric discharge of 75,000 volts for forty-five minutes in a vacuum at -196°C. Upon warming to room temperature, the peak intensities decreased by 90%. Figure 16 was recorded using gains 10 times those used to record Fig. 5.

The spectra of the samples treated by electrical discharge were qualitatively different from the spectrum of ground flour (compare figures 8 and 15). That is, free radicals produced by electric discharge appear to be different from those produced by grinding. If it is assumed that the electric discharge produces the same type of free radicals in flour as it does in dry protein, then it can be concluded from these experiments that the free radicals produced by grinding do not result from the abstraction of hydrogen atoms.

Figure 15

The ESR spectrum (recorded at -196°C) of the free radicals produced by subjecting outgassed flour at -196°C to a high voltage electrical discharge for 45 minutes.

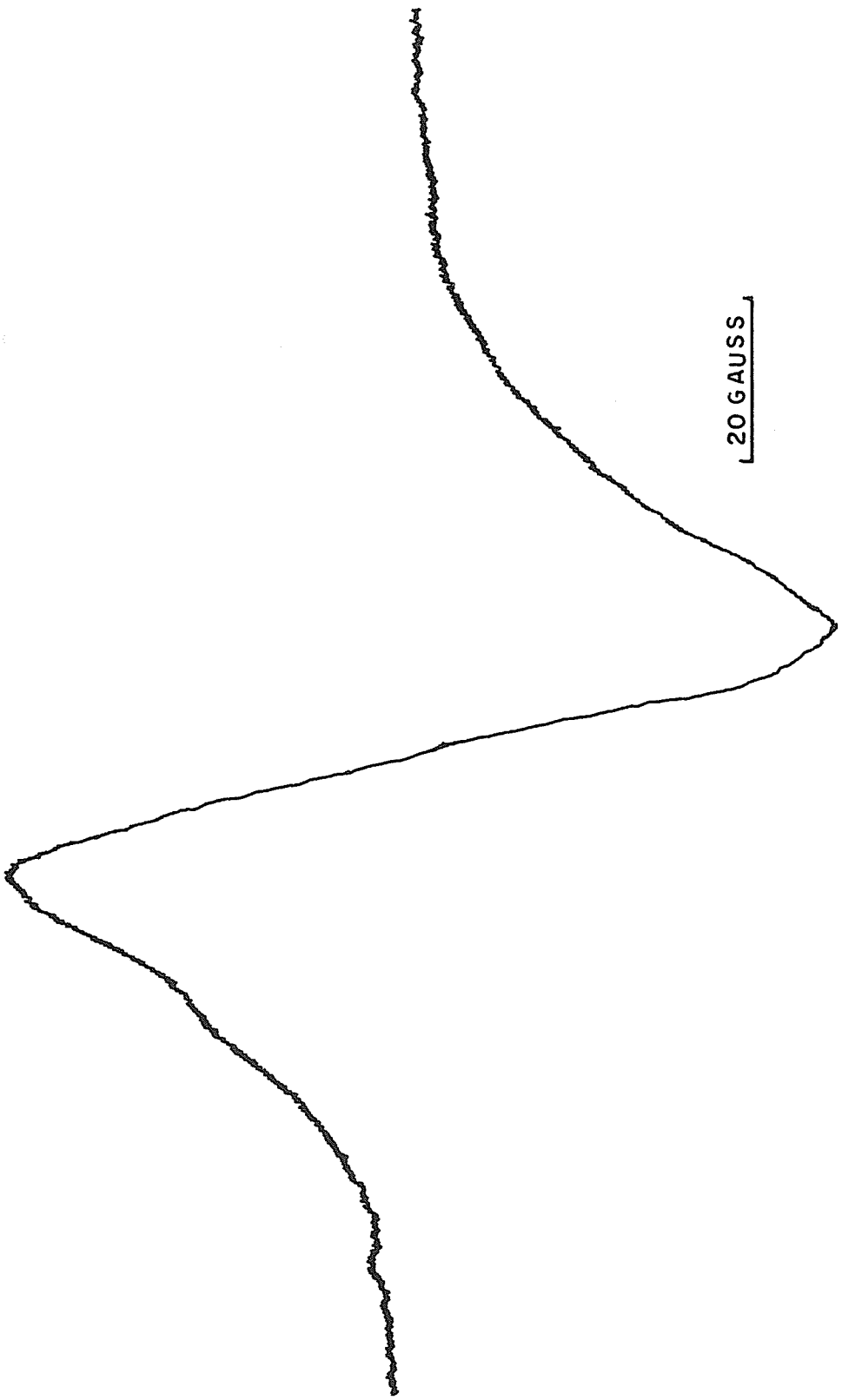
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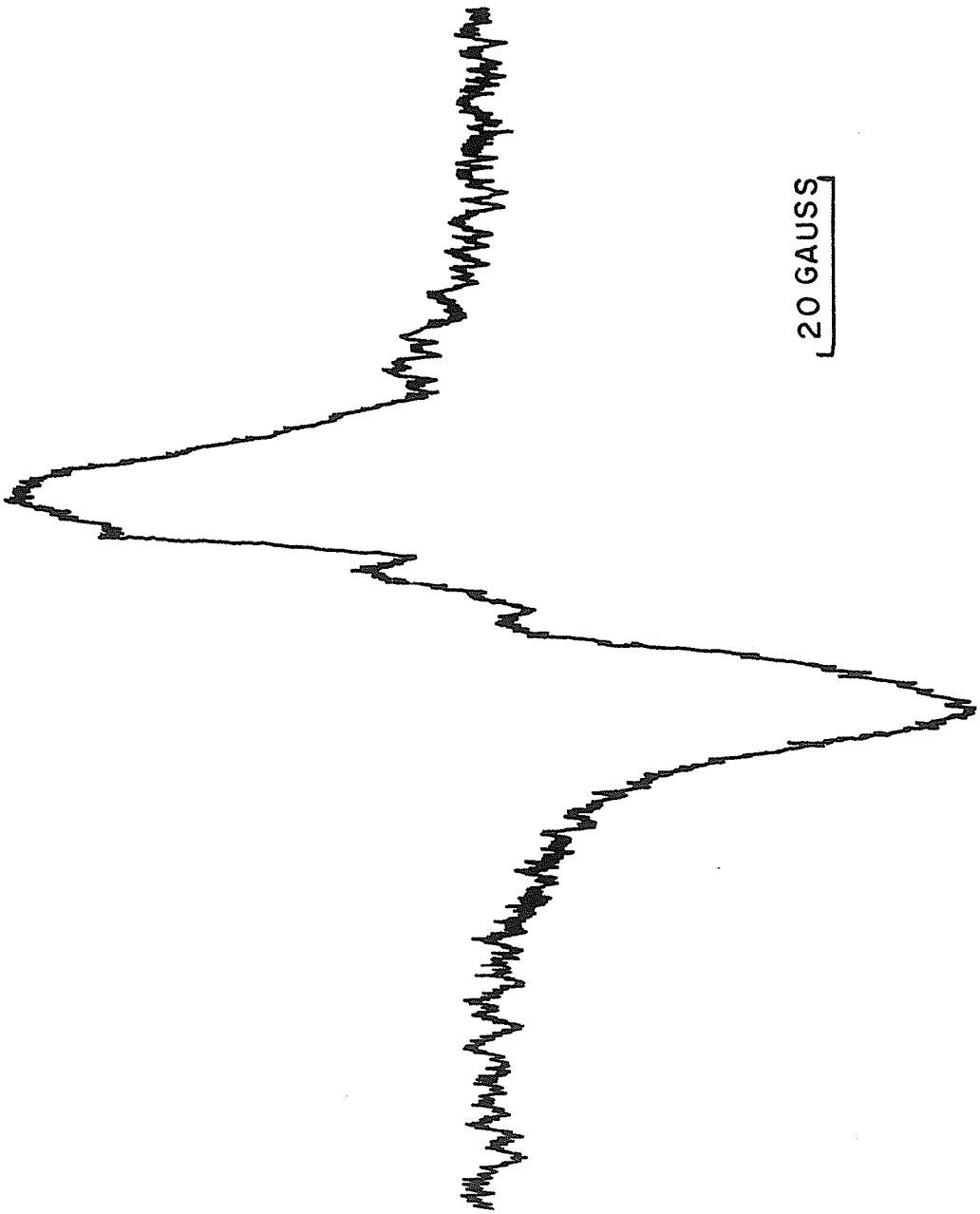
Figure 16

The ESR spectrum (room temperature) of the free radicals remaining in the flour sample which had been outgassed, subjected to an electrical discharge at -196°C and subsequently warmed to room temperature.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Free Radicals Produced in Flour and Gluten by γ-Irradiation

The production of free radicals by γ-irradiation of flour and gluten was studied to determine if these free radicals were the same as those produced by grinding and if the same free radical is produced in flour and in gluten.

The flour and gluten were irradiated for various periods under vacuum at -196°C in long quartz tubes. After irradiation, the upper portion of the quartz tube was annealed with a flame to remove the ESR signal of irradiated quartz. After cooling, the tube was inverted and the sample was shaken into the annealed portion of the tube which was then used to determine the ESR spectrum.

Figures 17 and 18 show the results that were obtained. The spectra of flour and gluten, recorded at -196°C, were almost identical both quantitatively and qualitatively and appear as broad singlets. Upon warming to room temperature, the signals became narrower and the intensities in both cases decreased by approximately 30% (Fig. 19). The flour signal remained slightly weaker than the gluten signal.

The free radicals produced by γ-irradiation are quite stable. Free radical concentrations remained essentially constant for several months under vacuum storage at room temperature. In air, there was a slow but continuous decay with time. After one week the intensities decreased by approximately 80%.

In another experiment, the flour sample (14% moisture content) was irradiated for one hour in an open vessel. The spectrum of the resulting free radicals is shown in Fig. 20. Although the concentration of free radicals in this sample was low, the existence of radicals was readily detectable. This irradiated sample was subsequently completely covered

with distilled water and the rate of decay monitored by observing the loss of intensity of the ESR spectra with time. It was observed that the free radicals remained quite stable under these high moisture conditions. These observations appear to be contrary to the findings of Lee et al. (13, 14, 15) who reported that the presence of water inhibited the production of free radicals by γ-irradiation.

The work of Redman et al. (11, 12) indicated that the radicals produced by ball-milling flour at -196°C in a vacuum are similar to those produced by γ-irradiation of flour under similar conditions. Results of our study showed that the free radicals produced by grinding flour in a vacuum at -196°C are inert to water. Oxygen, on the other hand, is known to be a very efficient free radical scavenger. It is possible that the results obtained by Lee et al. (13, 14, 15) could result from an inefficient removal of atmospheric oxygen from flour.

Figure 17

The ESR spectrum(recorded at -196°C) of the free radicals produced by γ-irradiating outgassed flour at -196°C.

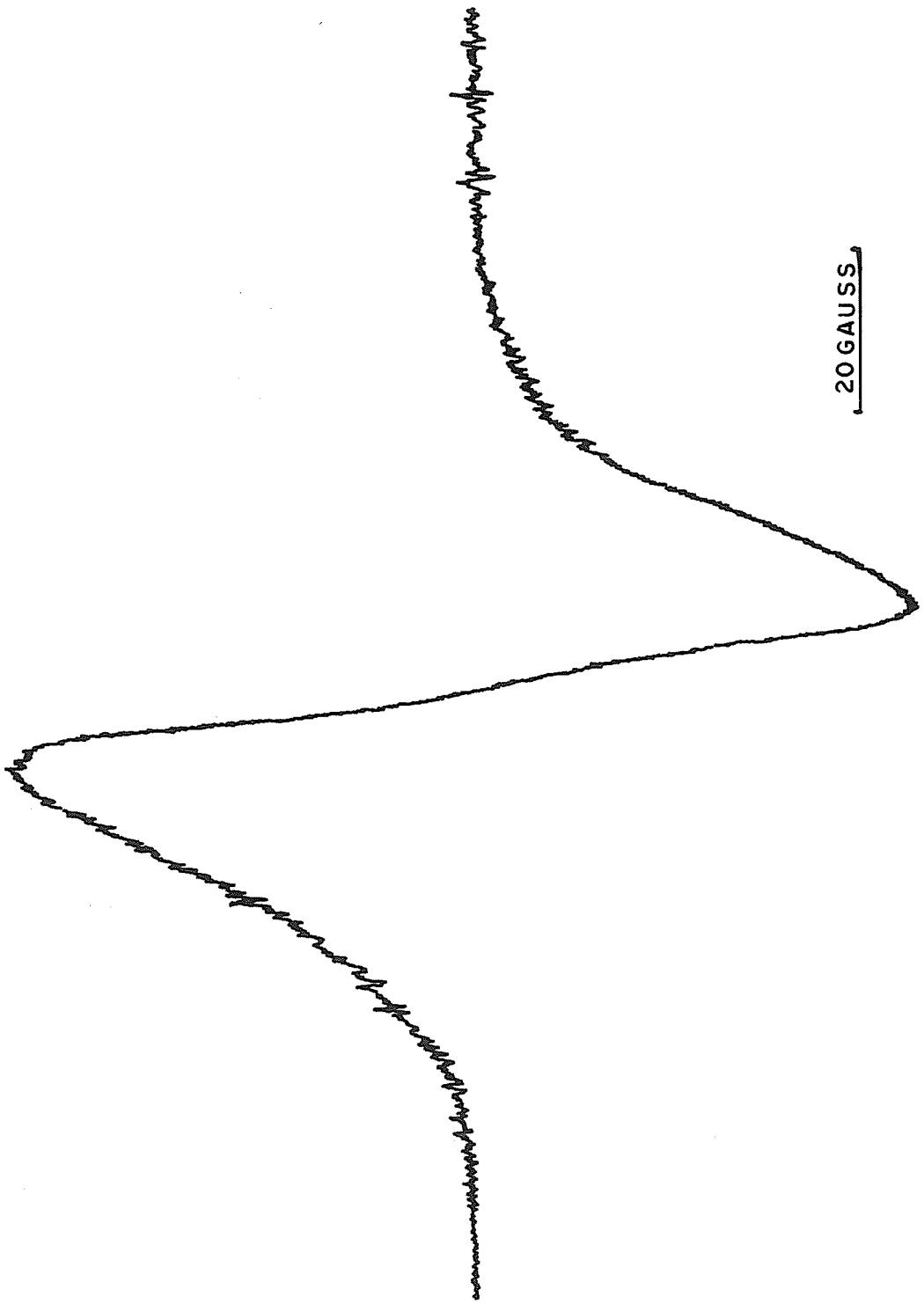
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Figure 18

The ESR spectrum (recorded at -196°C) of the free radicals produced by γ-irradiating outgassed gluten sample at -196°C.

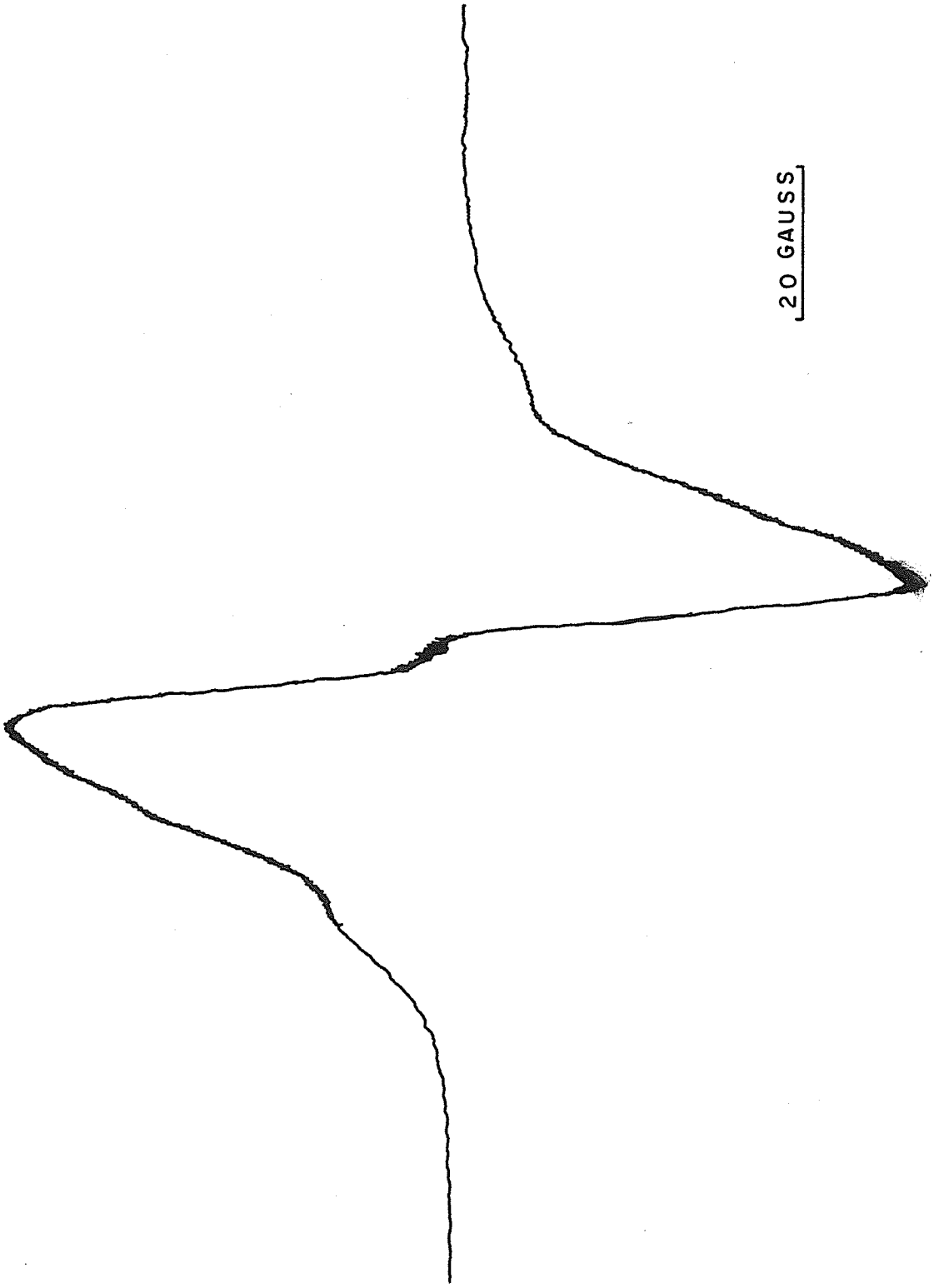
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Figure 19

The ESR spectra of the free radicals remaining in the gluten and flour samples after warming to room temperature. The sample had previously been outgassed and γ-irradiated at -196°C.

flour

gluten

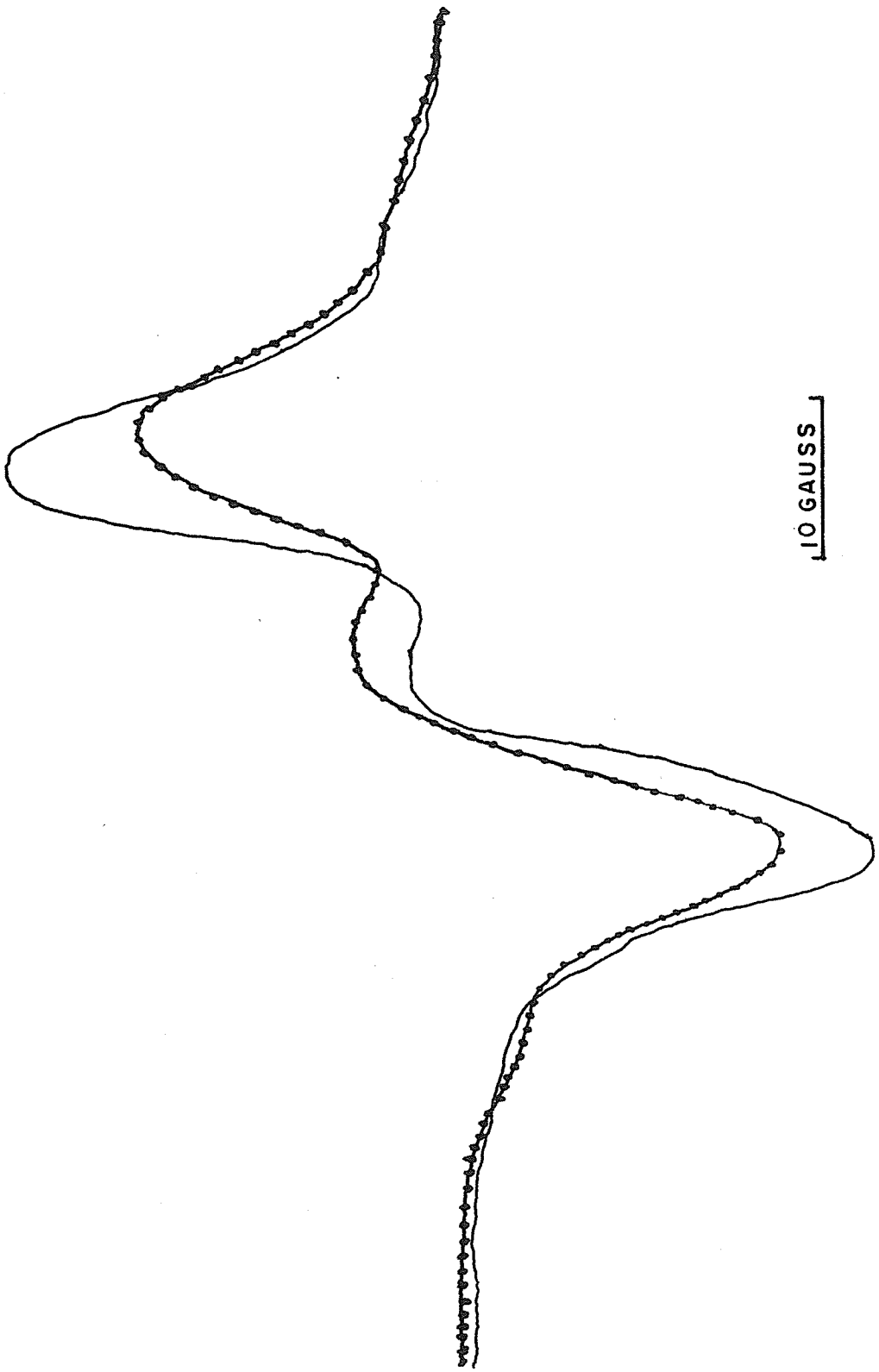
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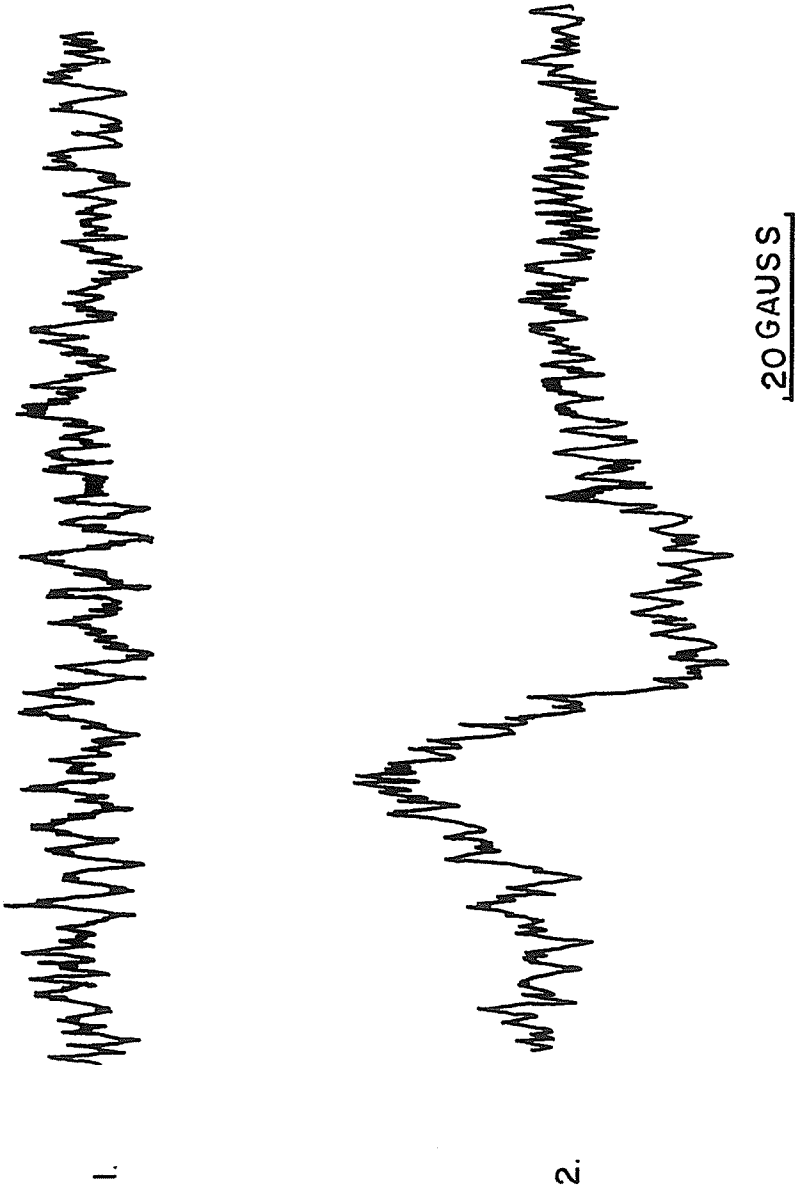
Figure 20

1. The ESR spectrum of flour (14% moisture content) prior to γ-irradiation.
2. The ESR spectrum (room temperature) of the same flour after brief γ-irradiation.

   Gain was $.5 \times 10^6$ in both cases.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1.
2.

Effects of Various Chemicals on Free Radicals in Flour Produced by Grinding

Experiments discussed in the previous section have provided some information on the stability of the free radicals produced by grinding flour and some of its constituents. Identification of these free radicals would be the next significant step in the study of the technological importance of these chemical species.

It is relatively easy to identify the free radical species in a pure compound with the ESR spectrometer. However, in complex systems, where several different types of free radicals might be present, the resulting spectrum recorded by the ESR spectrometer gives little or no information on the nature of the free radicals in the sample. Under such situations the usefulness of the ESR spectrometer in this regard is limited, and it functions only as a tool for quantitating the total free radical concentration.

To identify specific free radicals in a complex mixture, it is necessary to use a variety of indirect techniques. One experimental approach is to use substances which react rapidly, specifically and irreversibly with the free radicals to yield some product that can be isolated and identified qualitatively and quantitatively. Usually it is necessary to use several of the so-called free radical scavengers to obtain complete identification. It is sometimes possible to apply several different techniques which may overlap and complement each other. This experimental approach was therefore applied to the study of free radicals produced in flour by grinding. The results are discussed in the sections that follow.

The scavengers that were selected for the study are: tritium sulfide, sulfur dioxide, dibenzylnitrone and 2-methyl-2-nitroso

butanone-3. In addition to the experiments with these materials, this section will also deal with a more detailed study of the effect of water vapor on the free radicals in flour produced by grinding.

Effect of Tritium Sulfide

Recently Riesz et al. (21, 22, 23, 24, 25) have used tritium sulfide (T2S) to study the distribution of free radicals in γ-irradiated ribonuclease, chymotrypsin, insulin and lysozyme. Their studies indicated that,although the tritium label was distributed over a number of different amino acids, the distribution gave qualitative and quantitative estimates of the free radical concentration. On the basis of this information, two experiments were performed using T2S to attempt the identification of the free radicals produced by ball-milling flour and gluten in a vacuum at -196°C.

The two experiments (one using laboratory prepared gluten and the other on flour) were run concurrently. Two one-gram samples were placed in shaking bulbs equipped with pyrex break seals and outgassed.

Figures 21 and 22 show the ESR spectra of the free radicals produced by grinding flour and gluten in a vacuum at -196°C. The T2S was introduced into each sample container through a break seal and allowed to react for one hour at room temperature. After this time no trace of the original free radicals could be detected with the ESR spectrometer (Fig. 23).

To determine the reaction of tritium with the flour and the gluten, the samples were first treated to remove the readily exchangeable tritium by the procedure of Riesz et al. (21-25) and then hydrolysed by the procedure of Tkachuk et al. (18). The resulting amino acids were

Figure 21

The ESR spectrum (recorded at -196°C) of the free radicals produced by ball-milling outgassed flour at -196°C for 1.5 hours. Gain 5×10^5.

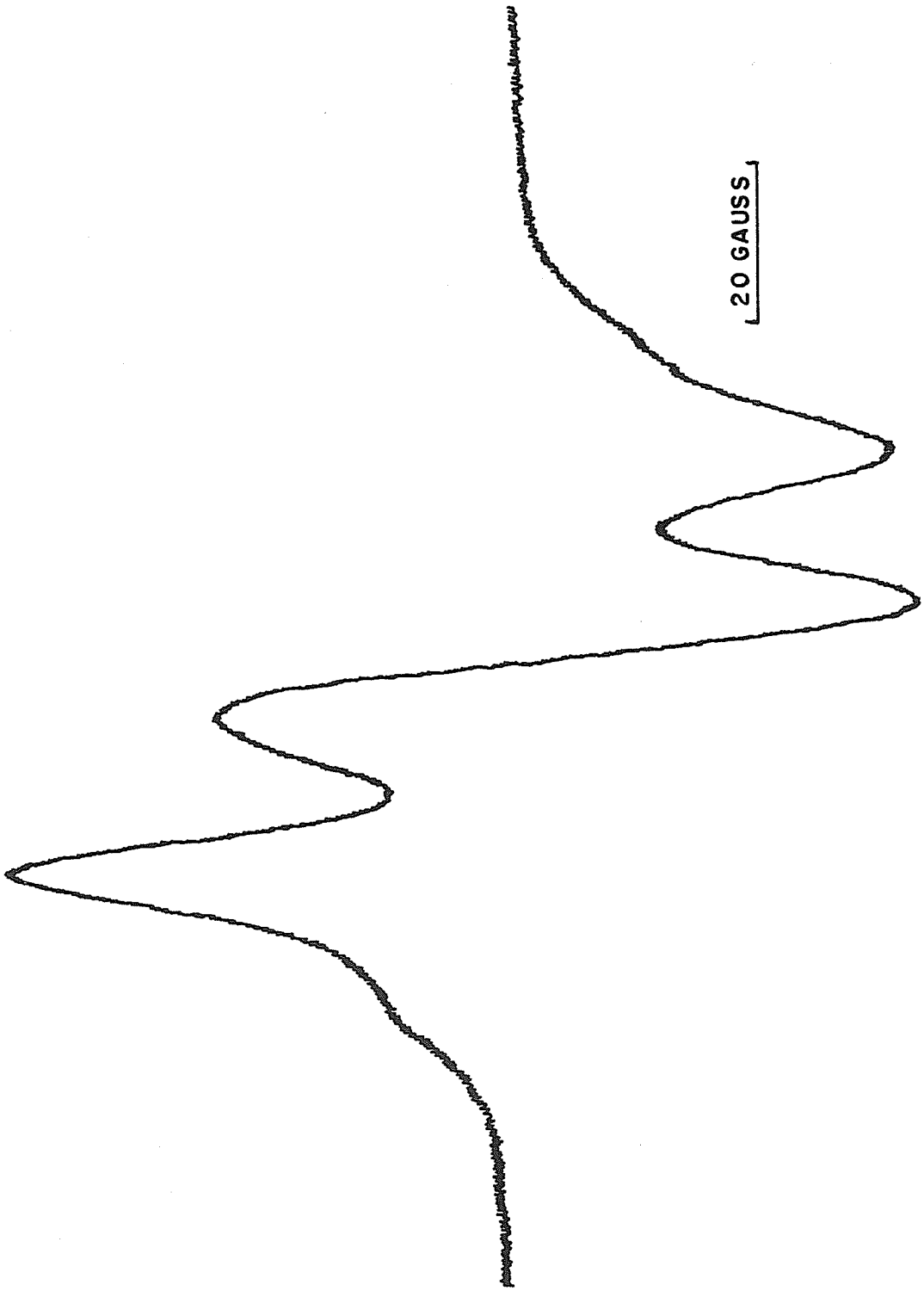
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Figure 22

The ESR spectrum (recorded at -196°C) of the free radicals produced by grinding outgassed gluten at -196°C for 1.5 hours. Gain 5×10^5.

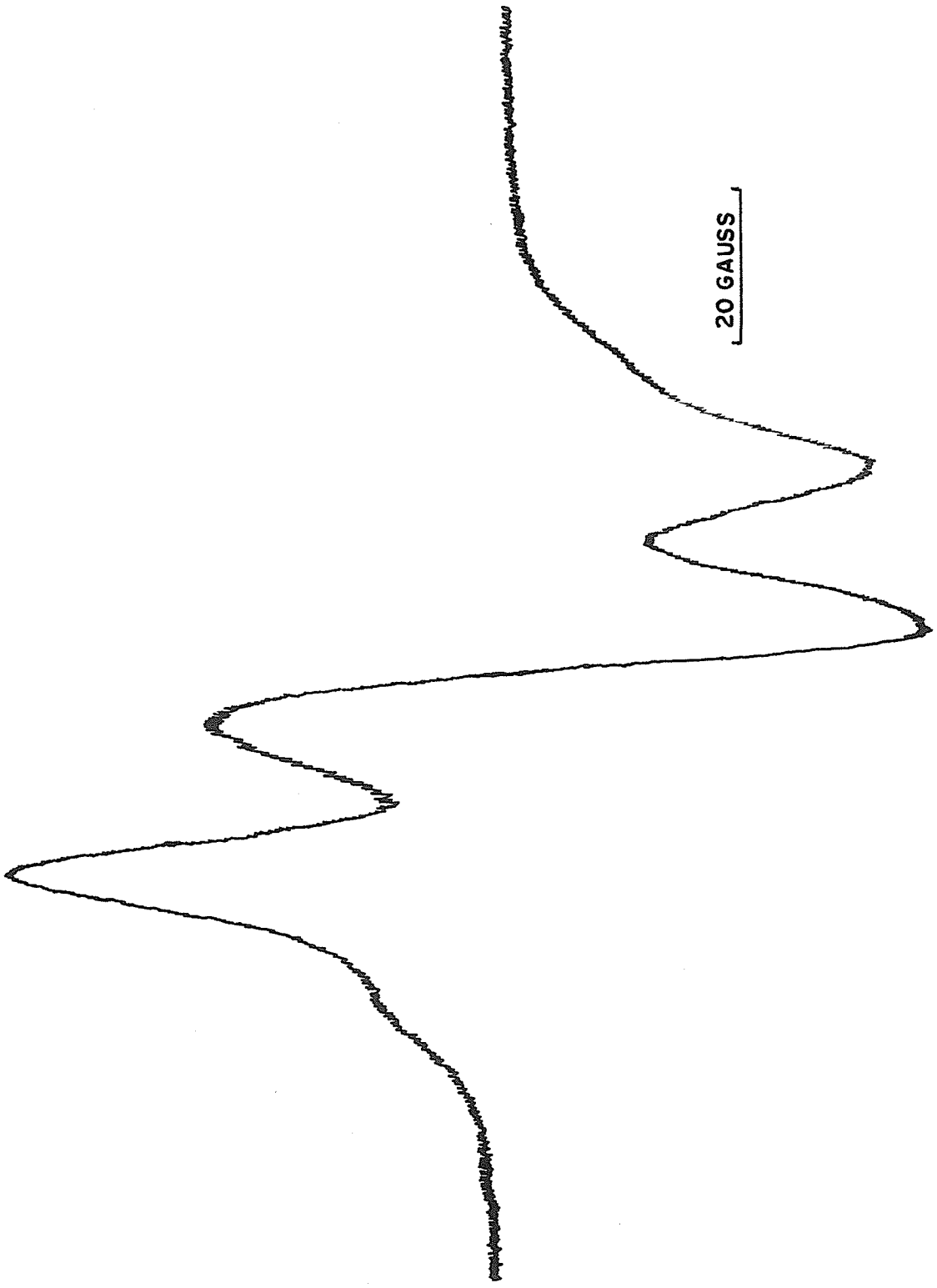
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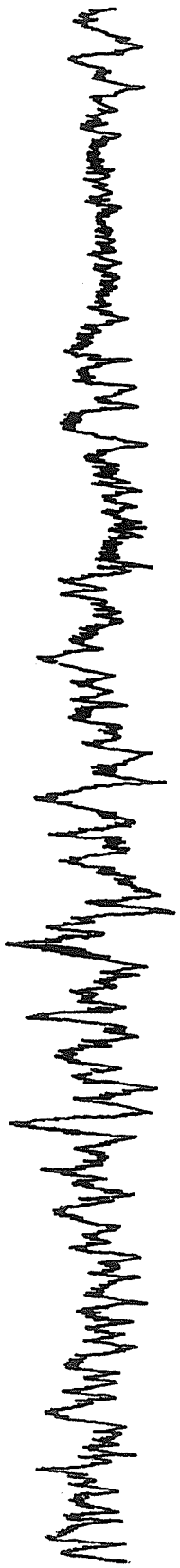
Figure 23

The ESR spectrum (room temperature) of the flour and gluten samples 1 hour after the introduction of tritium sulfide. Gain 2.5×10^6.



determined by radioautographic techniques. Control samples, not ground, were treated similarly.

No localized centers of activity could be detected on the x-ray plates after seven days exposure for any of the samples studied. The hydrolysate showed a very low amount of activity on the scintillation counter.

These results may be explained in either of three ways:

1) The concentration of free radicals in flour and gluten was too low to give a measurable activity after the reaction with T2S.

2) The amount of hydrolysate spotted on the paper prior to chromatography was too small and hence more time might be required for proper exposure.

3) The tritium that adds to the free radicals is lost through isotope exchange in subsequent steps of analysis after the addition of T2S.

Effect of Sulfur Dioxide

A recent investigation (26, 27) has shown that it is possible to establish a heterogeneous equilibrium in which gaseous sulfur dioxide adds to free radicals trapped in hydrocarbon polymers. This equilibrium is rapid and reversible. At room temperature, the equilibrium lies well to the side of the product radicals. Ayscough et al. (27) found that the addition of sulfur dioxide to γ-irradiated polypropene, and subsequent warming to room temperature transformed the original asymmetric spectrum into a symmetric signal. They postulated that the resulting radical was $RS^{\bullet}O_2$. The same authors reported that the spectrum was not altered when the sample was exposed to air and therefore it was

concluded that oxygen did not add to $RS^{\cdot}O_2$ or displace sulfur dioxide.

Good et al. (26) have compared the effectiveness of SO_2 and oxygen to scavenge $C^{\cdot}H_3$ free radicals. They found that the addition of SO_2 occurred at about the same rate as the addition of oxygen although SO_2 required a slight activation energy.

On the basis of the results on the ability of sulfur dioxide to add to the free radicals, it was decided that the effect of sulfur dioxide on the free radicals produced by ball-milling should be investigated as a possible means of identifying these free radicals.

The effectiveness of SO_2 to scavenge the free radicals produced by ball-milling flour in a vacuum at -196°C was studied employing the techniques used in the experiments with tritium sulfide. Varying amounts of reagent grade sulfur dioxide were fed into the glass bulb containing the freshly ground flour. The ESR spectra of flour samples before and after doping with SO_2 at -196°C, and of the sulfur dioxide treated sample after warming to room temperature are shown in Figs. 24, 25, 26 and 27. Although SO_2 was unreactive at -196°C it reacted readily at higher temperatures. ESR spectra showed that the free radical concentration decreased noticeably in a short period of time and to zero within a few hours at room temperature.

The ESR spectrum did not show any evidence of the formation of $RS^{\cdot}O_2$ radicals such as were obtained when sulfur dioxide reacted with irradiated polypropene.

The appreciable decrease in the intensity of the ESR spectrum of ground flour upon warming to room temperature after addition of sulfur dioxide indicates that it is a good scavenger for radicals in flour. However, since this reaction did not produce the $RS^{\cdot}O_2$ type of radical

Figure 24

The ESR spectrum (recorded at -196°C) of the free radicals produced by ball-milling flour in a vacuum at -196°C for 1.5 hours. Gain 2.5×10^5.

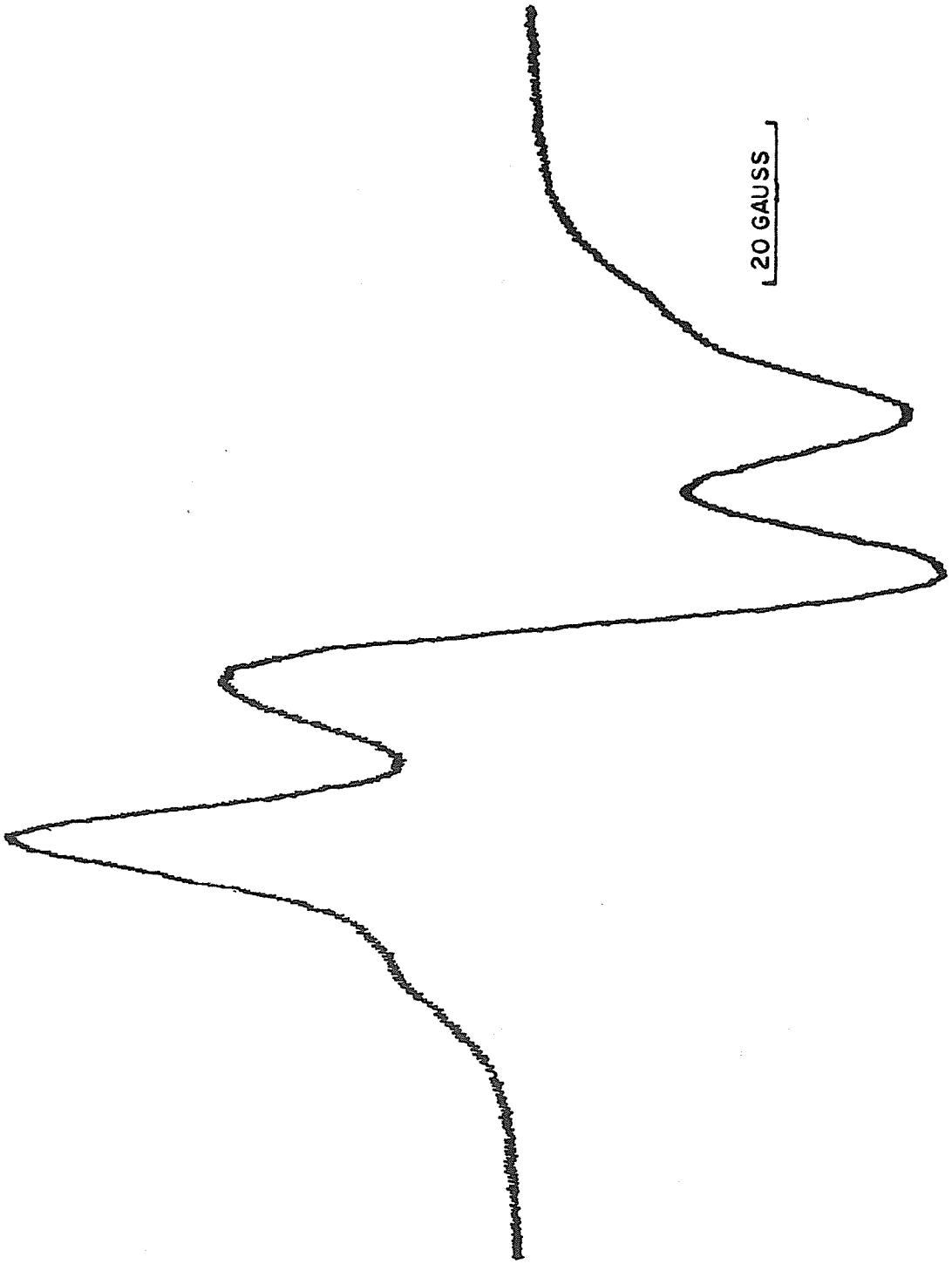

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Figure 25

The ESR spectrum (recorded at -196°C) of ball-milled flour after the addition of SO_2. Gain 2.5 x 10^5.

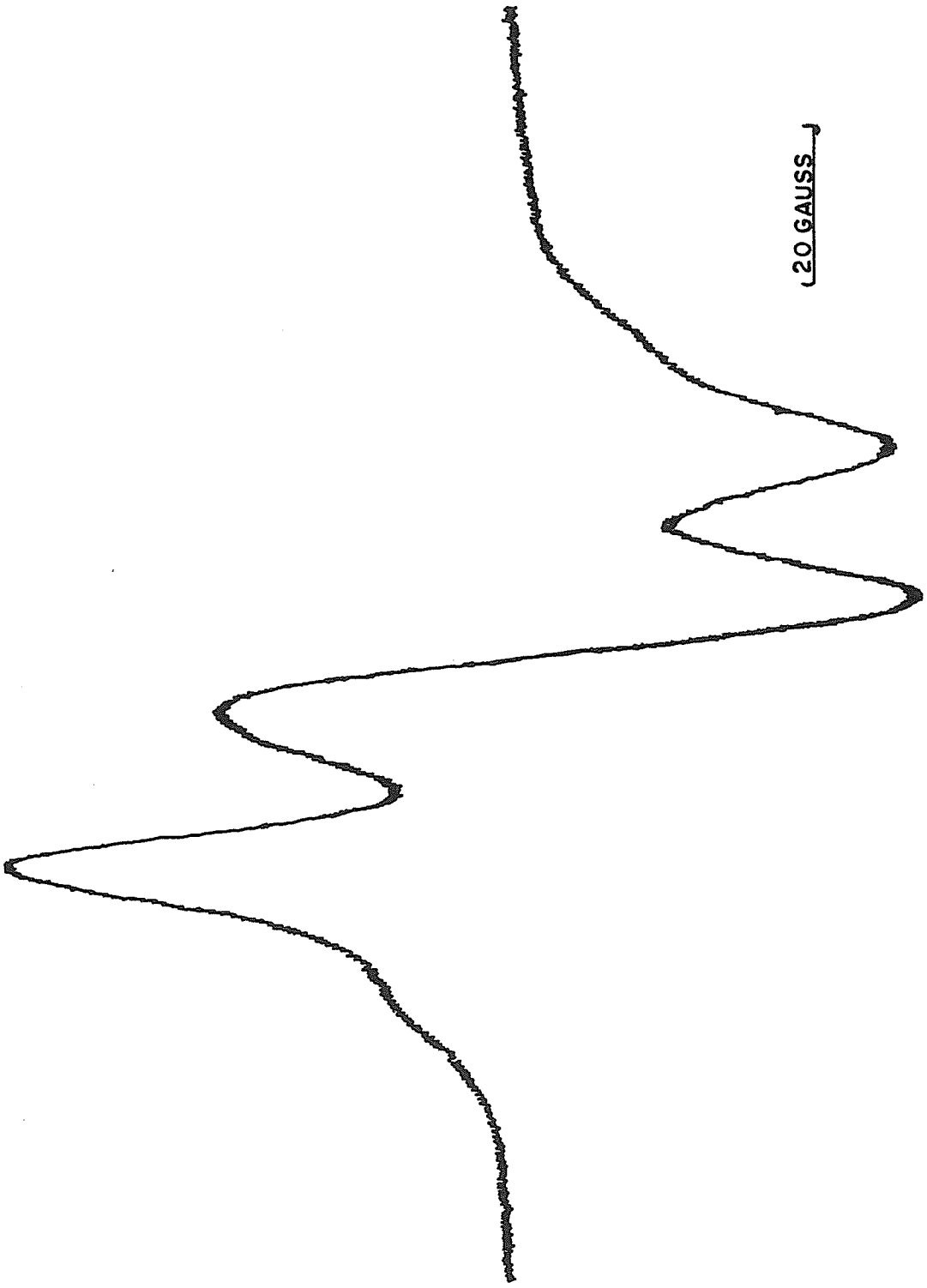
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Figure 26

The ESR spectrum (recorded at room temperature) of ball-milled flour shortly after warming to room temperature. Gain 10×10^5.

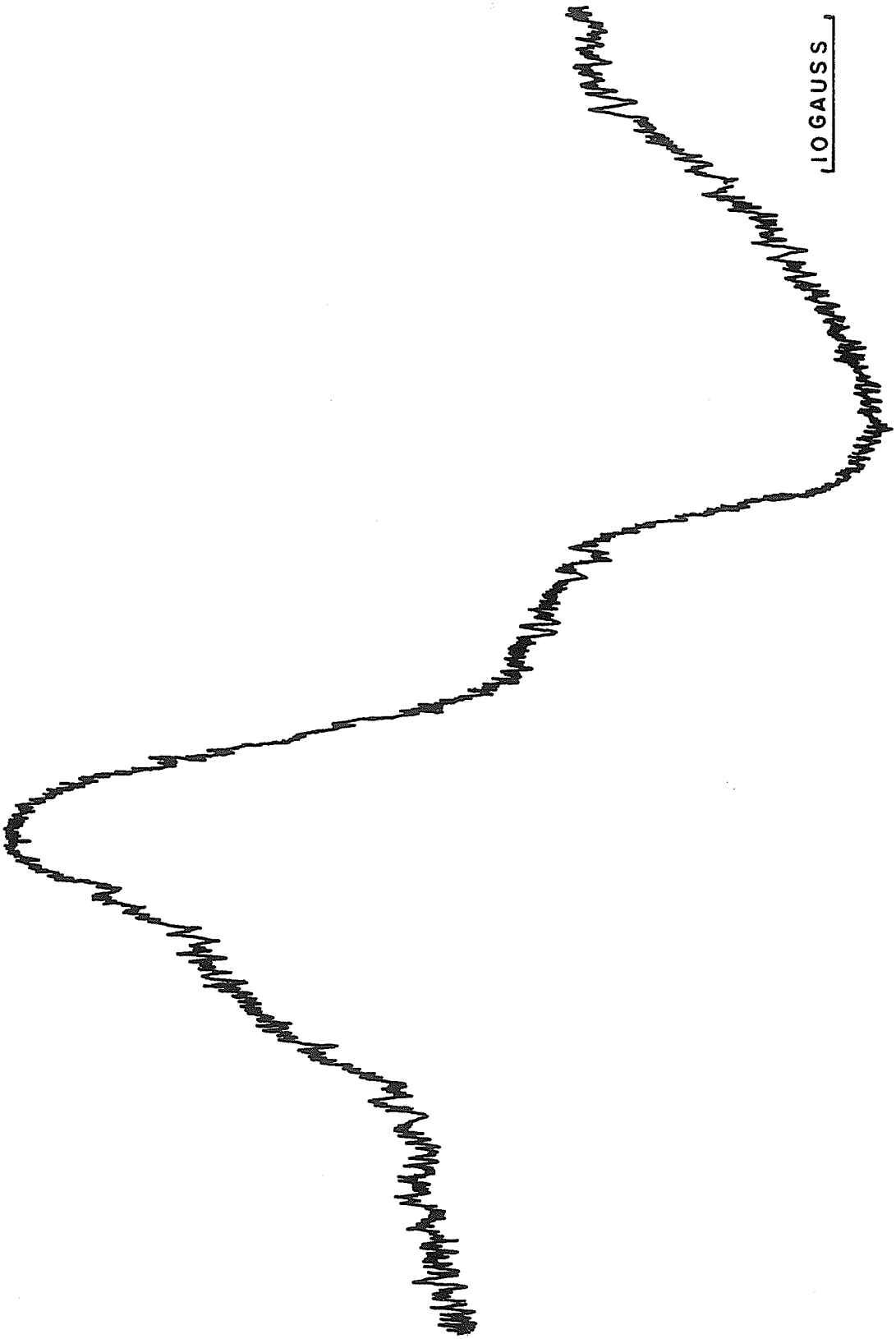
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Figure 27

The ESR spectrum (room temperature) of ball-milled flour after one hour at room temperature. Gain 2.5×10^{6}.

proposed by Ayscough et al. (27) for polypropene, it was concluded that subsequent reactions of this radical in flour yield a product that is nonparamagnetic.

Effect of Nitrone Compounds

In 1967 Imamura and Inamoto (28, 29, 30) reported that free radicals would react with a 1, 3 addition mechanism to the C or O atoms of a nitrone to yield a stable nitroxide free radical. Shortly thereafter Janzen and Blackburn (31) investigated the feasibility of applying phenyl-t-butyl nitrone, dibenzylnitrone and others to the study of unstable free radicals in solution using ESR techniques to monitor the reaction

$$R^{\circ} + C_6H_5-CH=\overset{O^-}{\underset{+}{N}}-C(CH_3)_3 \longrightarrow C_6H_5-\overset{H}{\underset{R}{C}}-\overset{O^{\circ}}{N}-C(CH_3)_3$$

The hyperfine interaction of the radical adduct in solution has certain characteristic features which are sensitive to the detailed structure of the added free radical groups. The information obtained from these features can then be used to identify the trapped radical. 2-Methyl-2-nitrosopropane (32), diphenylnitrone (29, 30), methylenenitrones (33), dibenzylnitrones and phenyl-t-butylnitrone have been shown to trap free radicals in solution to give stable nitroxide free radicals.

Although there are no references in the literature of attempts to trap free radicals in solids using solid nitrones, it was felt that an experiment designed to examine the effect of some nitrones on the free radicals produced in flour would be worthwhile, but no interaction was expected.

Dibenzylnitrone was used in our experiments because it appears to be, on the basis of nucleophilic properties, the most reactive nitrone of a group which could be readily synthesized.

The dibenzylnitrone was synthesized using the method of De La Mare et al. (34). Recrystallization of the crude product from benzene-petroleum ether gave a product with the expected melting point.

The nitrone (0.25 gm) and flour (1.0 g) were mixed, evacuated and ball-milled at -196°C. The ESR spectrum measured at room temperature was quite different from the analogous spectrum for flour (Compare Figs. 28 and 8).

The nature of these free radicals in the ground mixture of flour and dibenzylnitrone was not investigated further.

Effect of Nitroso Compounds

Like nitrones, nitroso compounds are also able to trap unstable and highly reactive free radical species by reacting with them to yield stable nitroxide radicals. These nitroxide radicals are characteristic of the original free radical. For example, if R° is a reactive free radical, and if the nitroso compound used is 2-methyl-2-nitroso-butanone-3, then the following reaction that would take place is:

$$R^\circ + (CH_3)_2 - \underset{\underset{O}{\|}}{\overset{\bullet}{C}}{}^{N} - C(O) - CH_3 \longrightarrow (CH_3)_2 - \underset{\underset{\bullet O}{|}}{\overset{\bullet}{C}}{}^{N-R} - C(O) - CH_3$$

The hyperfine coupling of the R° group on the nitrogen is characteristic of this group.

Most nitroso compounds function very effectively in neutral aqueous, organic, or mixed solvents. At low pH the effectiveness of the nitroso compound to form a nitroxide is inhibited (35, 36).

The effectiveness of solid nitroso compounds to scavenge free radicals produced in solid matrixes like flour has not been investigated.

The experimental procedure used here was similar to that used with the nitrone discussed in the previous section. The results indicated that free radicals produced by grinding a mixture of flour and 2-methyl-2-nitroso butanone-3 were qualitatively and quantitatively identical to the free radicals produced in the flour under the same conditions (Compare Figs 29 and 8). The nitroso compound does not interact with the free radicals produced in flour by grinding.

Effect of Water Vapor

Lastly the effects of water on the free radicals produced in flour by ball-milling were studied.

Two different experimental approaches were used: 1) addition of O_2 free water to an outgassed flour prior to ball-milling at -196°C, and 2) addition of O_2 free water after ball-milling in a vacuum.

Both experiments showed that the free radicals produced by ball-milling were not affected by the presence of water, (Compare figs. 30, 31 and 32). As noted in an earlier section, water also had no effect on the free radicals produced by γ-irradiation. All spectra were recorded at the same gains.

Figure 28

The ESR spectrum (room temperature) of the free radicals produced by ball-milling an outgassed mixture of flour and dibenzylnitrone at room temperature for 1.5 hours.

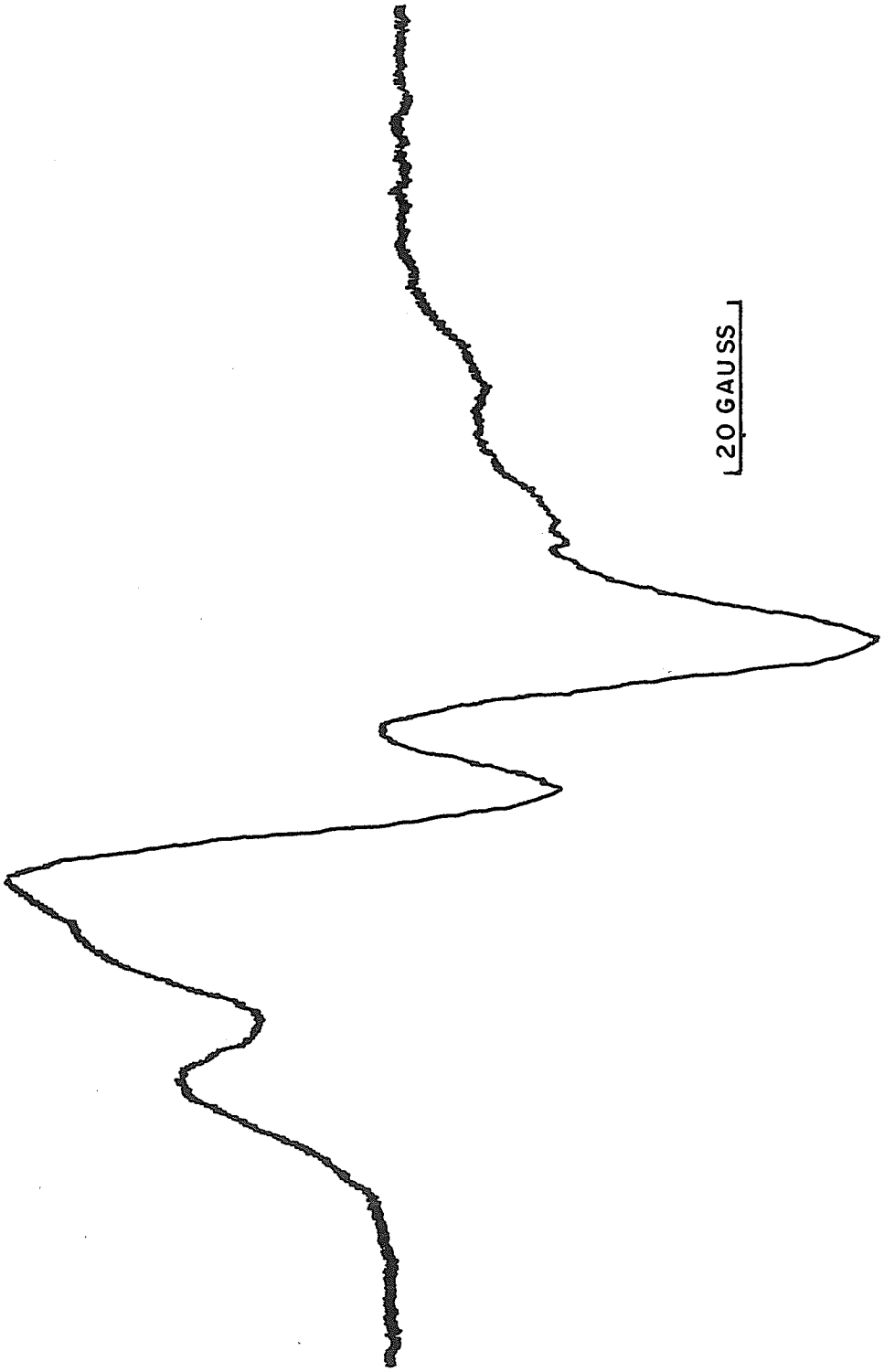
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Figure 29

The ESR spectrum (room temperature) of the radicals produced by grinding an outgassed mixture of flour and 2-methyl-2-nitroso-butanone-3 at room temperature for 1.5 hours.


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Figure 30

The ESR spectrum (room temperature) of the free radicals produced by grinding outgassed flour at -196°C for 1.5 hours.

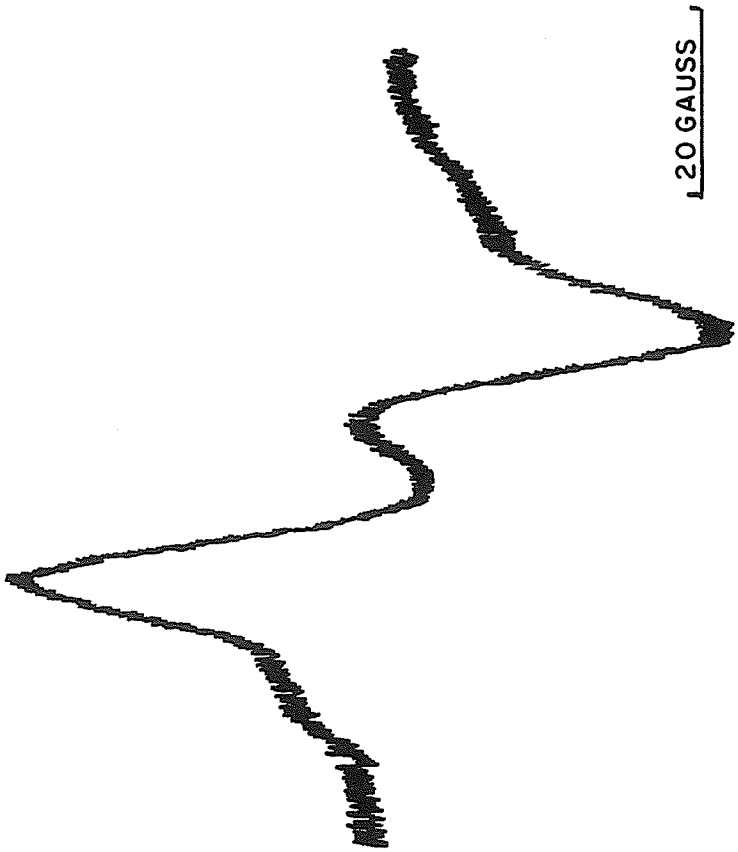
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Figure 31

The ESR spectrum (room temperature) of the free radicals produced by adding O_2-free water after an outgassed flour sample had been ground for 1.5 hours at -196°C.

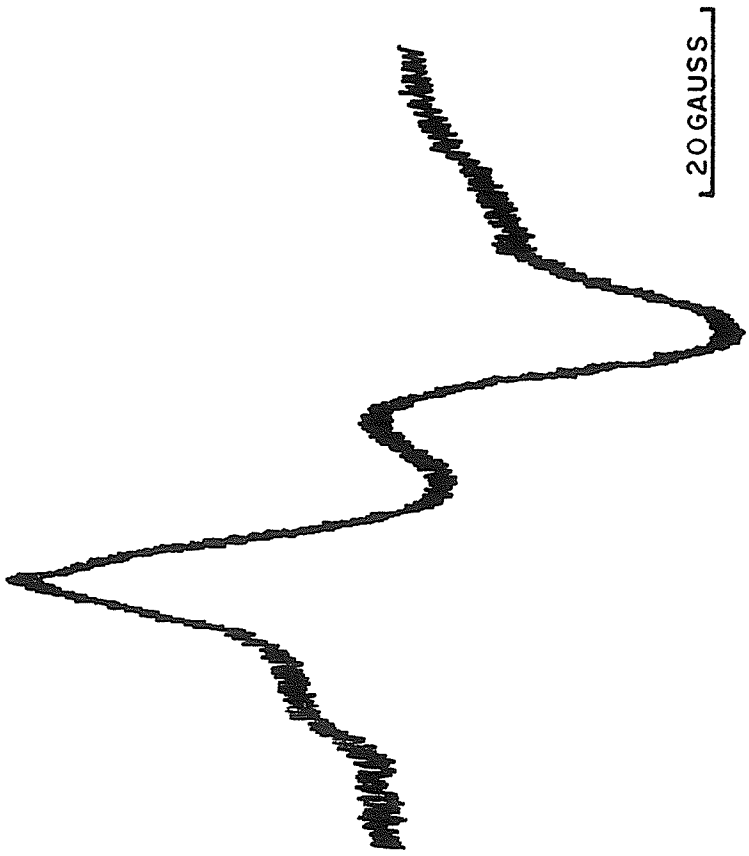
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Figure 32

The ESR spectrum (room temperature) of the free radicals produced by grinding an outgassed flour sample which had been doped with O_2-free water prior to grinding at -196°C for 1.5 hours.


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GENERAL DISCUSSION

The original objective of this thesis project was to verify or reject the hypothesis that free radicals are produced in dough during mixing. Presumably these free radicals would result from mechanical scission of covalent bonds, and could subsequently be involved in mechanical development of dough. Previous attempts to obtain direct ESR evidence using the most sensitive spectrometer available at the University of Manitoba were repeated. Results for six different doughs mixed at two speeds in three different atmospheres, were negative.

Attempts to obtain indirect evidence on the presence of free radicals in dough using free radical scavengers and monomers that polymerize in the presence of free radicals were also unsuccessful. In spite of the negative results, further studies on dough are warranted. Two possible new approaches can be used. The first is the use of flow techniques so that doughs can be examined in an ESR spectrometer while it is subjected to high shear stress in the spectrometer sample tube. A special spectrometer cell, attached directly to the mixer, would be required for this experimental set up. Another experimental approach would be to use isotope scavengers such as oxygen-18. This approach would require a mass spectrometer for analytical purposes.

Because of these negative results, the work on dough was abandoned in favor of further studies of the free radicals in flour that can be produced by grinding and γ-irradiation. The major portion of this thesis dealt with results of these studies.

Free radicals can be readily produced in flour by grinding. A large proportion of the free radicals produced by this action decay quite rapidly during storage at room temperature after grinding at -196°C,

but a relatively strong ESR signal persisted after relatively long periods of storage. It is quite possible that the highly reactive primary free radicals, produced by grinding at -196°C, react to form more stable radicals. Both the primary and secondary free radicals react rapidly with oxygen and sulfur dioxide but are quite inert in the presence of moisture (up to about 14%). The observation that water was ineffective as a scavenger of the free radicals produced by grinding is the most significant result of this study.

The production of free radicals by grinding that react quickly with oxygen might be important in relation to flour maturing. However, this possibility requires further study. Flour from the experimental Buhler mill examined by ESR as soon as possible after milling showed no evidence of free radicals.

Some of the results obtained in the present study are essentially the same as those obtained by Redman et al. (11, 12). The present study extends the publishing work to include the effects of post-grinding storage.

Free radicals can also be readily produced in flour by γ-irradiation. This was first examined in some detail by Lee et al. (13, 14, 15). Results of the present study differ from those of Lee et al. (13, 14, 15) in one very important aspect. These workers reported that the free radicals that they examined were readily scavenged by water at fairly low (below 14%) moisture levels. In contrast, the present study showed that the free radicals produced by γ-irradiation were quite stable in the presence of water. Redman et al. (11, 12) speculated that the free radicals produced by grinding were identical to the free radicals produced by γ-irradiation. The present study showed that both types

of radicals are quite stable in the presence of water at normal flour levels. The results of Lee et al. (13, 14, 15) could be explained on the assumption that the flour samples that they used contained traces of oxygen which is a highly effective scavenger of the free radicals that are produced by grinding or γ-irradiation.

After the conclusion of the present study, an unpublished report was made available to the writer by Dr. Evans of the U.S.D.A. Western Regional Laboratory on the presence of free radicals in a sample of U.S. hard red spring wheat. A number of different samples of the variety Manitou grown in the University of Manitoba experimental plots were examined by ESR spectrometry. There was no signal that would indicate the presence of free radicals. It appears that the presence of free radicals in wheat depends on the area of growth. Further studies are required to determine the significance of the observations of Dr. Evans.

SUMMARY AND CONTRIBUTIONS TO KNOWLEDGE

1. No evidence was obtained to support the hypothesis that free radicals are produced in doughs during mixing.

2. Free radicals may be produced by ball-milling flour, gluten and starch in a vacuum at room or at liquid nitrogen temperatures.

3. The ESR spectra of ground flour and gluten resemble one another more closely than those of ground starch.

4. Free radical concentrations in ball-milled gluten were higher than those of similarly treated starch and flour.

5. The free radicals produced by ball-milling appear to be a result of the scission of covalent bonds rather than X-H type bonds.
X - oxygen, nitrogen, carbon or sulfur.

6. The ESR spectra of γ-irradiated flour and gluten resemble each other closely.

7. Tritium sulfide, sulfur dioxide and oxygen effectively scavenge free radicals produced by either treatment.

8. Dibenzylnitrone and 2-methyl-2-nitrosobutanone-3 are ineffective scavengers in their crystal form.

9. Water vapor did not appear to have an effect on the production or stability of the free radicals.

10. In conclusion, the extensive studies carried out in attempting the identification and characterization of the various free radicals have made this thesis the best current review of modern techniques which may be applied in studies dealing with biological free radicals.

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