A NEW POWDERED AGENT FOR FLOUR MATURING[1]

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ABSTRACT

The preparation, chemical nature, and properties of a new maturing agent, azodicarbonamide, and its reduction product, biurea, are described. Data are presented to show that azodicarbonamide and its premixes have excellent stability. A postulated reaction mechanism of this new product in maturing flour is discussed. Treatment levels ranging from 2 to 45 p.p.m. of azodicarbonamide are required to accomplish maturing, the amount depending on the grade of flour. Baking data are given showing the effects of flour treatment with azodicarbonamide alone and in combination with benzoyl peroxide or bromate. The effects of azodicarbonamide on dough properties are illustrated by different physical dough testing methods. Ease of application of this maturing agent is demonstrated in commercial mill trials. Azodicarbonamide does not accelerate the onset of rancidity in flour. Natural or enrichment vitamins are unaffected by azodicarbonamide. Recovery data for biurea and radiotracer residue studies with azodicarbonamide are presented. Pharmacological and toxicological studies establish its safety.

The importance of flour improvers has been generally recognized for a long time in the milling and baking industries. For the last 13 years chlorine dioxide (7) has been the maturing agent of choice in the mills. As is well known, chlorine dioxide is applied in the gaseous state to flour and is therefore only suited for flour treatment in the mill. The performance of this gaseous improver has been quite adequate in many respects, but this method of treatment is a somewhat cumbersome procedure requiring complex apparatus. It was felt that a powdered maturing agent would present certain obvious advantages, particularly from the purely mechanical aspect of application to the flour. Additional requirements for such an improver, besides the already existing criteria for a gaseous flour maturing agent, were 1) that the compound be completely stable over long periods of time, both in its pure form and in the form of a suitable premix, and 2) the powdered agent and its premix not increase the ash content of flour.

The purpose of this paper is to describe such a product (4) and to provide information about its characteristics and its use.

Material and Methods

Preparation and Description of Azodicarbonamide. Azodicarbonamide is a yellow crystalline solid which melts with decomposition above 180°C. It is nonexplosive, nonflammable, nonirritating, leaves no resi-

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due on ignition, and contains no heavy metals.

Azodicarbonamide has the following structure:

$$H_2N-\overset{\overset{\displaystyle O}{\|}}{C}-N=N-\overset{\overset{\displaystyle O}{\|}}{C}-NH_2$$

and is prepared by the oxidation of biurea:

$$H_2N-\overset{\overset{\displaystyle O}{\|}}{C}-NH-NH-\overset{\overset{\displaystyle O}{\|}}{C}-NH_2,$$

which itself is made by coupling two urea molecules together.

Azodicarbonamide is a remarkably stable solid which can be used to treat flour directly, or more conveniently is diluted with an inert filler such as starch. By use of a 10% premix,[3] great accuracy of flour dosage can be attained and even small flour streams can be treated using conventional feeding devices for adding the premix to the flour.

Baking Procedure. All laboratory baking tests were made either by the straight "pup" method or the sponge dough procedure. The tests were carried out essentially as given in *Cereal Laboratory Methods* (1), using a nonbromate, noniodate type of yeast food in all but one of the bakes.

Results and Discussion

Mechanism of Reaction. According to the most generally accepted theory of flour maturing (8,9), it is believed that sulfhydryl groups on the protein molecules are involved in the improvement of the physical properties of flour by maturing agents. Presumably, these –SH-containing substances in flour are cysteine residues of the protein components and are oxidized to disulfide linkages. It has been found experimentally that azodicarbonamide will react easily with –SH-containing compounds, for example, cysteine, which is converted quantitatively in about 20 min. in aqueous solution to cystine, the azodicarbonamide being reduced to colorless biurea in the process. Biurea and cystine were identified by m.p. and m.m.p. with the corresponding authentic samples. Thiele (10) found in a somewhat similar reaction that the action of hydrogen sulfide on azodicarbonamide yielded biurea. It is postulated that azodicarbonamide exerts its maturing action on flour by a reaction analogous to the reaction with cysteine.

It was shown experimentally that azodicarbonamide does not react

[3]A 10% azodicarbonamide premix with starch diluent is presently marketed under the name MATUROX, a registered trademark of Wallace & Tiernan Inc.

with flour in the dry state but does react rapidly when flour has been wet with water as in the practice of making dough. In these experiments, several samples of flour treated with the optimum dosage for this flour of 8.25 p.p.m. azodicarbonamide were used. The reaction rate was followed by a chemical spot test which depends upon the liberation of iodine from an acidified potassium iodide solution by azodicarbonamide. Once flour is wetted with water, reaction of the azodicarbonamide with flour constituents is rapid, with final quantitative reduction to biurea. Unlike potassium bromate, azodicarbonamide does not require fermentation to bring about reaction but begins to react immediately after the flour is wetted. By 30 min., virtually all the azodicarbonamide has disappeared. Longer times than this elapse in the dough stage of bread-baking; thus all azodicarbonamide would be reduced to biurea before the baking stage is reached. The rate of the disappearance of azodicarbonamide in flour after wetting is summarized in Table I.

TABLE I

REACTION RATE OF AZODICARBONAMIDE IN FLOUR ON WETTING

Sample No.	Time after Wetting of Flour	Observations on Applying Spot Test for Azodicarbonamide
	min.	
1	5	Bulk of azodicarbonamide still present
2	10	Estimated ½ azodicarbonamide left
3	15	Estimated ¼ azodicarbonamide left
4	20	Estimated ⅛ azodicarbonamide left
5	25	Few percent azodicarbonamide left
6	30	Trace azodicarbonamide left
7	45	No azodicarbonamide detectable

Limit of sensitivity = 0.1 p.p.m. azodicarbonamide

Recovery Studies. Since the evidence indicated that azodicarbonamide was reduced to biurea upon reaction with the flour on wetting, an analytical method was developed which was specific for biurea and was used to confirm that this was the residue in the finished bread. This analytical method was based on extraction of the biurea from dough or bread with water, removal of interfering substances, and finally, estimation by a spectrophotometric method.

Recovery data obtained with this method show that azodicarbonamide is converted quantitatively to biurea on reaction with flour upon wetting. Biurea is a very stable compound, resistant to heat and hydrolysis. It undergoes no further change, and the residue in the finished bread is biurea.

TABLE II

BIUREA RECOVERY IN BLENDED WHEAT PATENT FLOUR, TREATED WITH AZODICARBONAMIDE

AZODICARBONAMIDE ADDED	AZODICARBONAMIDE RECOVERY AS BIUREA	AZODICARBONAMIDE ADDED	AZODICARBONAMIDE RECOVERY AS BIUREA
p.p.m.	%	*p.p.m.*	%
150	85	10	100
150	92	10	108
30	85	10	83
30	88	5	121
30	113	3	117

TABLE III

BIUREA RECOVERY IN BREADS MADE FROM AZODICARBONAMIDE-TREATED FLOURS

BAKING PROCEDURE AND TYPE OF FLOUR	FORMULA	AZODICARBONAMIDE Added	AZODICARBONAMIDE Recovered as Biurea	BAKING PROCEDURE AND TYPE OF FLOUR	FORMULA	AZODICARBONAMIDE Added	AZODICARBONAMIDE Recovered as Biurea
		p.p.m.	%			*p.p.m.*	%
Straight dough—"lean"		150	93	Sponge dough—"rich"		150	92
(Blended wheat patent)		150	94	(Blended wheat patent)		150	86
		150	96			150	102
		30	90			30	99
		30	84			30	103
		30	98			30	102
		10	98			30	100
		10	84	(Clear)		30	108
		10	92	(Blended wheat patent)		10	107
		10	100			10	107
						10	106
						10	91
						10	89
						5	115
						5	98
						2	108

Tables II and III show the residue data for azodicarbonamide-treated flour and for bread made from azodicarbonamide-treated flours.

As can be seen, treatment was varied over a wide dosage range. Based on recovery experiments with biurea, the reliability of the analytical method was ± 20%, and biurea residues of the order shown in Tables II and III indicate quantitative recovery of the added azodicarbonamide as biurea.

Residue Studies with Labeled Azodicarbonamide.[4] Data from radiotracer experiments provided additional evidence that azodicarbonamide is reduced by the flour to biurea, which can then be recovered quantitatively from the finished bread. C^{14} azodicarbonamide[5] was in-

[4] These studies with tagged azodicarbonamide as well as the toxicological and biochemical studies described below were performed at Food and Drug Research Laboratories, Inc., Maspeth, N.Y.

[5] Material was synthesized by personnel of Wallace & Tiernan Inc. at the New England Nuclear Corporation, under their supervision.

corporated into a straight bread formula at a level of 10 p.p.m. based on the flour content. The carbon dioxide, which formed during the fermentation and baking of the bread, was collected and converted to barium carbonate which was then assayed for radioactivity. A bread sample was also crushed and dried and the evolved carbon dioxide trapped and converted to barium carbonate. C^{14} assays of the different gaseous samples showed that less than 0.1% of the original C^{14} was present in the evolved gases. Additionally, the bread slices were decomposed by combustion to carbon dioxide and the latter assayed for C^{14}. The dried bread crumbs had a specific activity equal to more than 99% of the amount of C^{14} added, indicating essentially no breakdown of biurea and complete retention of C^{14} biurea in the finished bread. It was concluded from these results that C^{14} biurea did not decompose during the fermentation or baking of the bread, or subsequent grinding and drying of the resulting material.

Stability of Azodicarbonamide. As can be seen from Tables IV and V, undiluted azodicarbonamide or premixes made with starch or

TABLE IV
STABILITY OF AZODICARBONAMIDE

Composition	Storage		Azodicarbonamide Remaining
	Time	Temperature	
	months	*°F.*	%
Azodicarbonamide in pure form	15	70	99.5
	24	70	100
	15	110	99.5
	24	110	99.5
10% Azodicarbonamide premix with corn starch	24	70	98
	15	110	95
	24	110	95

TABLE V
STABILITY OF AZODICARBONAMIDE IN FLOUR MIXTURES

Premix	Original Amount of Azodicarbonamide	Azodicarbonamide Remaining	
	(per 100 g. mixture)	13 Months	24 Months
	g.	%	%
Azodicarbonamide 5th middlings flour	30.0	100	99
	9.4	100	100
Azodicarbonamide untreated Bakers'	31.3	100	99
	9.3	100	100
Azodicarbonamide rye blend flour	32.2	96	98
	9.6	100	99
Azodicarbonamide medium rye flour	32.6	97	97
	9.8	98	98

flour show excellent stability over a long period of time.

In additional experiments, flour samples were treated with azodicarbonamide over a dosage range from 5 to 25 p.p.m. in order to determine whether the compound is stable in flour at normal treatment levels. The different samples were stored at room temperature and 110°F. for 36 and 100 days, respectively. Comparison of the stored samples with freshly treated samples by baking tests revealed that the maturing agent was still available in its original concentration.

Deterioration of Flour by Rancidity. It has been reported that the application of certain chemical flour improvers appears to reduce the effectiveness of the natural antioxidants present in flour (6), and flours treated in such a way develop rancidity much earlier than untreated flours. Azodicarbonamide does not react in the dry state, and therefore stored samples of flour treated with this maturing agent do not become rancid any faster than samples of untreated flour. In a test comparing untreated flour samples with flours treated with azodicarbonamide and chlorine dioxide, respectively, the samples were stored in tightly capped glass bottles in the dark and examined organoleptically for presence of rancidity at periodic intervals by a four-man panel.

The data in Table VI show clearly that azodicarbonamide-treated flour samples exhibit practically the same resistance to rancidity as untreated flour.

TABLE VI
EFFECT OF TREATMENT ON RANCIDITY DEVELOPMENT IN FLOUR
(FLOUR FIRST CLEAR)

Treatment				Moisture	Time at 110°F. to Develop Rancidity
Chlorine Dioxide		Azodicarbonamide			
p.p.m.	*g./cwt.*	*p.p.m.*	*g./cwt.*	%	*weeks*
Untreated control				12.95	23
22	1.0			12.87	8
28	1.25			12.81	7
33	1.50			12.91	7
		10	0.45	12.80	20
		15	0.68	12.91	21
		20	0.91	12.98	21
		25	1.13	12.82	21

Effect of Azodicarbonamide on Dough. The rheological properties of doughs made from flours treated with azodicarbonamide were compared with untreated and chlorine dioxide-treated flours via the following observations by physical dough testing methods: (a) dough handling characteristics during baking tests; (b) effects on the gluten fraction of flour; and (c) Chopin Extensimeter tests.

(a) Extensive observations were made of the handling properties of doughs during a long series of baking tests. Azodicarbonamide-treated flours consistently yielded drier, more cohesive doughs than chlorine dioxide-treated flours, and these drier doughs were superior in their machining properties. In one typical test, doughs produced from clear flours, containing 10 to 15 p.p.m. azodicarbonamide, were compared with doughs obtained from the same flours, treated with 20 p.p.m. and more of chlorine dioxide. The first-mentioned doughs were judged superior because they showed less stickiness, tolerated slightly higher absorption, and were more pliable. In another test, a patent flour at a level of 7.5 to 10 p.p.m. azodicarbonamide yielded a dough that was smoother and tighter and had better machinability than doughs formed from 4.5 to 13.5 p.p.m. chlorine dioxide-treated patent flour. All of the above treatments covered the optimum range for these particular flours.

(b) Comparison was made of the washed glutens from untreated flour and from flour treated either with azodicarbonamide or chlorine dioxide. The gluten was tested for extensibility and elasticity by stretching and filming into a sheet. Next, the gluten was worked into a ball and placed on a ½-in. mesh wire screen in a 110°F. hot-air cabinet. The amount of gluten that passed through the screen overnight and before the gluten hardened was measured. In most cases, the bulk of the glutens from the chlorine dioxide-treated flours flowed through the screen, whereas a good portion of the glutens from the azodicarbonamide-treated flours remained on the screen. The conclusions from these results were that the doughs from azodicarbonamide-treated flours produced firmer, less extensible, more rubbery, and bolder glutens than the doughs from chlorine dioxide-treated flours, which in turn yielded better glutens than the untreated flours. In general, a strong gluten is desired since it gives an indication of better handling and machining characteristics of the doughs.

(c) Experiments were made using the Dalby (2) modification and technique of the Chopin Extensimeter, which is designed to measure the extensibility of gluten as well as its gas-retaining powers. A typical curve with a Bakers' Patent comparing chlorine dioxide and azodicarbonamide with an untreated flour sample is shown in Fig. 1. These curves show an increased resistance to expansion of the bubbles as well as a decrease in the extensibility of the films formed from doughs made with azodicarbonamide and chlorine dioxide-treated flours. The effects were similar for both types of flour treatment. This test also distinguishes between the action of azodicarbonamide and potassium bromate on flour, since the latter agent does not change the properties of dough under the conditions of this test.

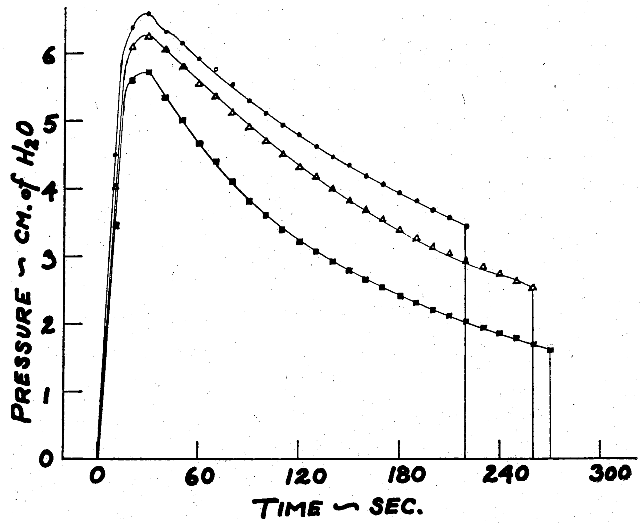

Fig. 1. Chopin extensimeter curves for Bakers' Patent Flour. Treatment: (top — dots) 10 p.p.m. azodicarbonamide; (middle — triangles) 18 p.p.m. Dyox; (bottom — squares) untreated.

Tolerance to a wider range of absorption was also illustrated by baking tests. In these experiments the effect of azodicarbonamide and chlorine dioxide treatment of flours were compared at various absorptions, ranging from 60 to 66%. The results showed that both flours treated with the two maturing agents responded to different water absorptions to about the same degree. The volume-weight ratio and the grain and texture of the different bread loaves were all similar. Untreated flour showed less tolerance over the same absorption range as judged by the final bread characteristics.

Flour-Maturing Tests with Azodicarbonamide. Comprehensive experimental baking tests extending over many crop years have shown that azodicarbonamide is an effective maturing agent for flours of different wheats and seasons. The amount required depends on the flour grade, as has been previously observed with other maturing agents. Clear flours require more oxidative treatment than patent flours. Depending upon the grade of flour, azodicarbonamide treatment of 2 to 45 p.p.m. was required in the samples tested.

Most of the evaluation of the azodicarbonamide requirement of different flour grades was carried out by baking straight-dough "pup" loaves. The results showed that this maturing agent was effective upon all grades of flour tested. The bread was characterized by increased loaf volume, finer grain, softer texture, and thinner cell walls. Likewise, the doughs showed improved machinability and gas-retention properties. The improvement of the bread characteristics obtained with azodicarbonamide treatment at least matched and, in some cases, was better than that observed for chlorine dioxide. The azodicarbonamide treatment imparted superior dough-handling properties. Typical examples of these baking tests are shown in Table VII.

TABLE VII
BAKING TEST EVALUATION OF AZODICARBONAMIDE TREATMENT ON DIFFERENT GRADES OF FLOUR ("PUP" LOAVES — STRAIGHT DOUGH)

Grade of Flour	Treatment				Absorption	Loaf Volume	Grain and Texture Score	Maturity
	Azodicarbonamide		Chlorine Dioxide					
	p.p.m.	*g./cwt.*	*p.p.m.*	*g./cwt.*	%	*ml.*		
Kansas Patent	Untreated control				61	790	96	Mature
(A)	2.5	0.11				855	98	Mature
			4.5	0.2		795	98	Mature
Kansas Clear	Untreated control				61	755	94	Green
(A)	15	0.68				800	96	Mature
			28	1.25		790	96	Mature
Kansas Patent	Untreated control				61	805	93	Mature
(B)	2	0.09				800	94	Mature
			2.2	0.1		790	95	Mature
Kansas-Clear	Untreated control				61	730	92	Green
(B)	16	0.72				830	95	Mature
			22	1.0		820	95	Mature
West Coast Clear	Untreated control				62	625	83	Green
	30	1.36				700	92	Mature
	40	1.81				765	93	Mature
			66.5	3.0		740	94	Mature

The photograph (Fig. 2) illustrates the characteristic differences that are brought about in patent flours by maturing with azodicarbonamide. There is a distinct increase in volume, and the improvement in grain is clearly evident.

The results of baking tests for the evaluation of azodicarbonamide-treated flours milled from different classes of wheat and blends of such wheats are shown in Table VIII. The data indicate that the maturing agent was effective in improving all the types tested. Whenever the flour samples had a greater need for oxidative treatment, the response

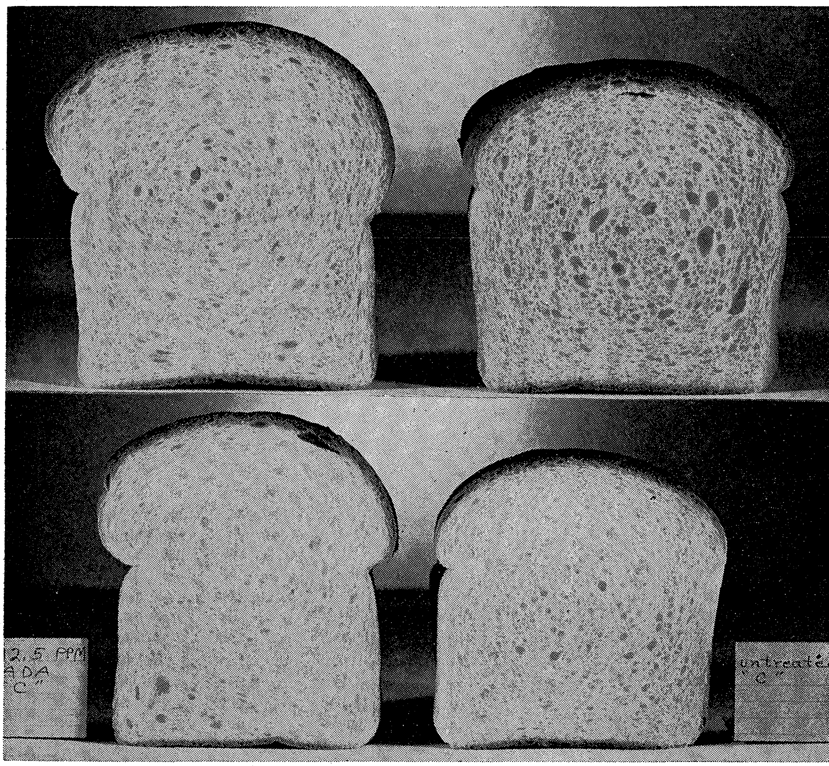

Fig. 2. Two untreated and treated patent flours, A and C. Untreated samples on right. 10 p.p.m. azodicarbonamide (ADA) (top left); 12.5 p.p.m. azodicarbonamide (ADA) (bottom left).

was more dramatic. Marked loaf-volume response and better grain and texture were obtained. Enhanced dough-handling properties were invariably observed.

Since there can be wide variations in the oxidation requirements of flours obtained from different crop years, a series of baking tests was carried out over the period 1955–1959 to determine whether azodicarbonamide was able to make up for these differences. The flours were baked by the sponge dough method. The maturing process equalized the dough and loaf characteristics of bread baked from flours from different crop years. Since no significant differences were observed, it is not necessary to report the details of all these tests. Instead, the data for two crops with considerable difference in oxidation requirement were selected and are shown in Table IX.

While an occasional standard Bakers' Patent may require up to 15 or 20 p.p.m. azodicarbonamide, as exemplified by 1956 Kansas

TABLE VIII

Baking Tests with Flours from Different Classes of Wheat ("Pup" Loaf — Straight-Dough Procedure)

Sample	Azodicarbonamide Treatment		Loaf Volume	Grain and Texture Score	Maturity
	p.p.m.	*g./cwt.*	*ml.*		
Montana Clear	Untreated control		650	82	Green
	20	0.91	850	97	Mature
Montana Straight	Untreated control		750	90	Green
	10	0.45	830	92	Mature
Kansas Patent	Untreated control		680	93	Mature
	5	0.23	720	95	Mature
Kansas Clear	Untreated control		670	88	Green
	20	0.91	760	93	Mature
Pacific Northwest Blue Stem	Untreated control		690	85	Green
	15	0.68	810	92	Mature
Northern Spring Bakers' Patent	Untreated control		790	95	Mature
	3.3	0.15	830	96	Mature

TABLE IX

Baking Tests with Flours from Different Crop Years (Sponge-Dough Procedure)

Flour	Treatment				Specific Volume	Grain and Texture Score
	Azodicarbonamide		Chlorine Dioxide			
	p.p.m.	*g./cwt.*	*p.p.m.*	*g./cwt.*	*ml./g.*	
1956-Kansas hard winter wheat, standard Bakers' Patent	Untreated control				5.52	95
	15	0.68			5.84	97
	20	0.91			6.05	98
			16.7	0.75	5.86	97
			22.2	1.0	6.01	98
1959-Kansas hard winter wheat, standard Bakers' Patent	Untreated control				6.04	96
	7.8	0.35			6.35	99
			11.6	0.52	6.13	99

hard winter wheat shown in Table IX, it is estimated that the azodicarbonamide treatment for most of the standard Bakers' Patents will average somewhat less than 10 p.p.m., as judged from the experience gained in all the baking evaluation.

Evaluation of Combinations of Azodicarbonamide and Potassium Bromate. Potassium bromate is an effective maturing agent, commonly added to flour regardless of whether or not it has been previously treated in the milling process with a maturing agent. The addition of potassium bromate takes place mainly in the bakeshop. For a long time, there has been discussion as to the need of this supplemental treatment at the baking stage of flours already matured at the mill. Many baking tests were carried out with combinations of azodicarbonamide and potassium bromate to determine whether azodicarbonamide is compatible with an additional bromate treatment. Chlorine dioxide-potassium bromate combinations were included in these tests, since they are generally used in practice today. An unbleached patent flour was baked by the sponge-dough method with and without the addition of a bromate-type yeast food. The data for this baking test are shown in Table X. The results of this series indicate not only that azodicarbonamide is compatible with potassium bromate, but also, since there may be some additive effect beyond that noted with other improvers, that the two treatments complement each other.

TABLE X
BAKING TESTS ON AZODICARBONAMIDE- AND BROMATE-TREATED FLOUR (ONE-POUND LOAF — STRAIGHT-DOUGH PROCEDURE)

Treatment					Loaf Volume	Grain and Texture Score	Maturity
Azodicarbonamide		Chlorine Dioxide		Arkady[a]			
p.p.m.	*g./cwt.*	*p.p.m.*	*g./cwt.*	%	*ml.*		
Untreated control				0	2,270	85	Green
				0.25	2,480	93	Mature
		11	0.5	0.25	2,680	94	Mature
10	0.45			0.25	2,690	95	Mature

[a] Yeast food containing 0.3% potassium bromate. This corresponds to 7.5 p.p.m. $KBrO_3$ per loaf.

Evaluation of Azodicarbonamide Treatment in Combination with Benzoyl Peroxide. Azodicarbonamide has no bleaching properties. However, bread baked from azodicarbonamide-treated flour gives the appearance of additional whiteness because of a finer texture and grain produced by simple change in the physical structure of the baked bread. Laboratory baking tests were conducted to establish the benzoyl peroxide dosages needed for optimal color removal with azodicarbonamide-treated flours. For example, one 84% Bakers' Patent required 1/4 oz. of 32% benzoyl peroxide per cwt. when used with azodicarbonamide. The same flour without azodicarbonamide but with 1 g. chlorine dioxide per cwt. required 1/16 oz. per cwt. less benzoyl peroxide.

The results of a number of such tests indicated that an average of 1/16 oz. or less of additional 32% benzoyl peroxide would be needed for each g./cwt. of chlorine dioxide omitted. In this way, the additional bleach normally imparted by chlorine dioxide treatment is matched and the final color of the flour standardized.

Commercial Flour Mill Trials. Commercial mill application followed by bakeshop results furnishes the final criteria for evaluation of the effectiveness of a new maturing agent. Two flour mill trials with bakeshop evaluation were conducted in order to study the applicability of azodicarbonamide to the two major types of wheat grown in the U.S.A., Northern spring and Southwestern winter wheat.

To summarize these tests, the flour maturing results attained by azodicarbonamide treatment were considered equal or slightly superior to those from chlorine dioxide treatment. No difficulties were experienced in application of the new material using existing mill-feeding devices. The azodicarbonamide-treated flour was able to produce very stable doughs, and the finished bread from these trials showed the physical improvement predicted by all laboratory tests.

Effect of Azodicarbonamide on Vitamins of Flour or Vitamins Added for Enrichment. A study was made of the effect of azodicarbonamide on natural vitamins of flour, on those supplied by enrichment, and on the vitamin content in bread made from these flours. Unbleached flour samples, both unenriched and enriched, were treated with 10 p.p.m. azodicarbonamide. Flour and bread samples were assayed[6] for their thiamine, riboflavin, and niacin content. The findings demonstrated that the addition of azodicarbonamide to natural and enriched flour has no destructive effect on the thiamine, riboflavin, or niacin content in the flour or in bread baked from these flours.

Pharmacology and Toxicology of Azodicarbonamide and Biurea. As has been outlined above, azodicarbonamide exerts its action on flour, is reduced to biurea during the process of making dough, and remains as biurea in the finished bread. In order to ascertain the safety of azodicarbonamide as a flour additive it was necessary to establish the pharmacology and toxicology of biurea.

An extensive study[7] was carried out which included: (a) 2-year feeding tests in rats and dogs with 100 to 1,000 times the anticipated level of biurea, resulting from azodicarbonamide treatment: these experiments included clinical observations on four generations of rats; (b) 2-year feeding tests in rats and dogs with bread made from flour treated with tenfold the anticipated amounts of azodicarbonamide including all the observations made under (a); (c) a 1-year feeding study in rats and dogs with massive levels of 5 and 10% biurea in the diet;

[6] See footnote 4.
[7] See footnote 4.

(d) a metabolism study in the rat using biurea tagged with C^{14} on both carbon atoms; (e) an *in vitro* study of the effect of simulated digestive juices on biurea; and (f) a study of the effect of azodicarbonamide treatment on the amino acids of wheat gluten.

These comprehensive chronic toxicity studies included clinical, biochemical, hematological, and gross and microscopic pathological examinations; the results showed that biurea is practically void of physiological effect at any dosage level. Even the attempts to produce some adverse pharmacological or physiological effects by feeding massive levels of biurea were negative except for physical effects arising from the limited solubility of this compound. The reproduction and lactation efficiency of the albino rats was examined during the chronic feeding period according to established procedures. From the offspring of three descendent generations of rats, observations were made similar to those made in the F_0 generation. The growth of the offspring and the indexes for the evaluation of fertility, gestation, viability, and lactation showed no adverse responses. The differences, if any, observed in these indexes between the test and control groups indicated no interference of biurea with the normal reproductive physiology in these animals. The conclusion drawn from these feeding tests is that azodicarbonamide and biurea are markedly free of toxicity.

The metabolism study, as outlined under (d), was intended to indicate whether biurea is absorbed by the gastrointestinal tract and if so, whether it was metabolized and by what organs of the body.

Oral administration of radioactive biurea to rats was chosen as the best available method for the study of this problem. A single dosage of 33 mg. of the material was used. This is a moderately heavy level which would be equivalent to the biurea residue in about 4 kg. of bread made from flour treated with the normal amount of azodicarbonamide. The findings indicated limited absorption of the compound with no detection of any tissue that had a special affinity for the compound. Complete clearance of radioactive material occurred a reasonable time after ingestion.

The susceptibility of biurea to hydrolytic or enzymatic attacks by gastric and intestinal juices was investigated by *in vitro* experiments. Biurea tagged with C^{14} was subjected to the action of simulated gastric and intestinal juices and subsequently separated by paper chromatography and counted. This study showed that biurea is not attacked by pepsin and trypsin, which are the two major digestive enzymes. These findings eliminate the possibility of an eventual breakdown of biurea in the digestive tract.

Finally, it had to be determined whether treatment of flour with azodicarbonamide destroyed or altered any of the amino acids which compose the protein molecules of gluten. Several flour samples were treated with azodicarbonamide, both at a normal and at a tenfold treatment level. A third sample containing the equivalent biurea concentration was included to account for any artifacts that might arise. Gluten samples were then prepared from these flours and subjected to both acidic and alkaline hydrolysis to liberate the amino acid components, which were separated by column chromatography with the fractions collected and analyzed according to established procedures (5).

The amino acid contents of all samples were considered to be within the normal range of analytical variability. The elution curves and the amino acid analysis were taken as criteria for comparison between the different gluten samples and the different hydrolytic cleavage methods used. Since there was no significant difference between the control and any of the treated gluten samples, it was concluded that azodicarbonamide treatment had no demonstrable effect on the amino acid constitutents of flour gluten.

The results of the pharmacological and toxicological studies, together with the amino acid analysis of gluten, were accepted as sufficient evidence to establish the safety of azodicarbonamide as a flour additive (3).[8]

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[8] In 1962 the Definitions and Standards of Identity for Cereal Flours and Related Products were amended to permit the use of azodicarbonamide as an optional ingredient within a quantitative limit of not more than 45 p.p.m., and a food additive regulation was issued by the Federal Food and Drug Administration covering its use.