# Reduction of the Azo Food Dyes FD&C Red 2 (Amaranth) and FD&C Red 40 by Thermally Degraded D-Fructose and D-Glucose

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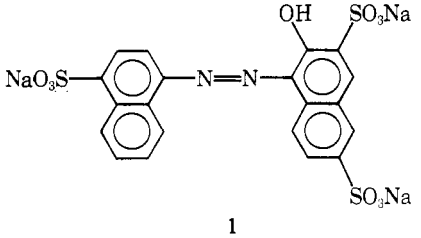
FD&C Red 2 (Amaranth) and Red 40 are reduced by aqueous D-fructose at 100, 77, 61, and 37.8° and by aqueous D-glucose at 100° to form a mixture of hydrazo and amine species. The half-reaction time, $\tau_{1/2}$, decreases with increasing pH. No reaction was observed with either sugar at room temperature for 40 days.

FD&C Red 2 (Amaranth) (1) and Red 40 (2) are monoazo dyes used extensively in foods to obtain a wide range of red hues. Both dyes are reported to be stable in the presence of common food ingredients. For example, "no appreciable change" in color is detected in 10% solutions of glucose, sucrose, citric acid, or acetic acid, or in solutions of pH 8 or less (McCormick, 1971). On the other hand, fading of Red 2 has been reported at temperatures above 135° (Stange Co., 1970), in the presence of metals in acidic solution (Lolua et al., 1967), and in the presence of ascorbic acid, particularly when the ascorbic acid is accompanied by $H_2O_2$ (Eisenbrand, 1973). McCormick (1971) claims that Red 2 does not fade in 10% solutions of invert sugar while Stange Co. (1970) reports a rapid fading when cane sugar in solution is hydrolyzed by citric acid or invertase.

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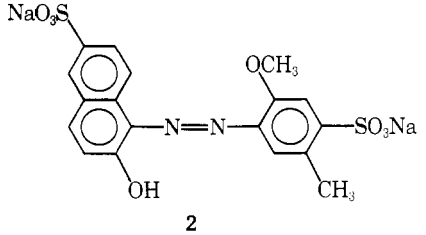


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Monoazo dyes are most readily degraded by either mild reduction to the corresponding hydrazo form (NH-NH) or complete reduction to two amines ($NH_2$, $H_2N$). Certain reducing agents are specific, e.g. $SnCl_2$ for direct, complete reduction, $Na_2SO_3$ for initial reduction to hydrazo followed by slow reduction to amines (Ogawa et al., 1971). Other reducing agents, such as metallic Fe, produce a mixture of hydrazo and amines (Lolua et al., 1967). Although both types of reduction have been reported for Red 2, there are as yet no literature data concerning the behavior of Red 40.

The present study was initiated to identify possible causes for observed color fading in several foods, particularly during storage at elevated temperatures. Because the chemistry of a finished food product is extremely complex, individual ingredients were selected for model studies. Reducing sugars, formed from sucrose during processing, were an immediate selection, in part because of the ambiguity of published data concerning dye stability with respect to invert sugar. Furthermore, many applications of food dyes involve processing temperatures of 60–100° or higher, a temperature range in which degradation of reducing sugars results in highly reactive species (Hodge, 1953; Fleming et al., 1968; Shaw et al., 1968). This report deals with the model system of dye-reducing sugar in aqueous solution.

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## EXPERIMENTAL SECTION

**Materials.** Red 2 was Food Materials Corp. 92% pure dye; Red 40 was Warner-Jenkinson 90% pure dye. Pfanstiehl provided D-fructose and D-glucose. Purity of sugars was confirmed by the absence of uv absorption and by TLC with BuOH–pyridine–$H_2O$ (6:4:3) as eluting solvent (Tu et al., 1960). Aldrich Chemical Co. supplied 5-hydroxymethylfurfural (HMF), diacetyl, acetoin (3-hydroxy-2-butanone), 1,3-dihydroxyacetone, glycolaldehyde, D-glyceraldehyde, acetaldehyde, and pyruvic acid, all used without further purification. Levulinic acid was obtained from Nutritional Biochemicals Corp. Pyruvic aldehyde was prepared by distilling acidic glyceraldehyde (Thornton and Speck, 1950). Triose reductone was obtained from glucose according to the method of Bauer and Teed (1955).

**Methods.** Stock solutions of Red 2 and Red 40 were prepared at concentrations of approximately $10^{-3}$ g/ml using distilled, deionized water. Dye concentrations in reaction mixtures were calculated from the visible absorption spectra. Molar extinction coefficients used were: $\epsilon_{522}$ (Red 2) = $2.78 \times 10^4$ l. cm$^{-1}$ mol$^{-1}$, $\epsilon_{505}$ (Red 40) = $2.81 \times 10^4$ l. cm$^{-1}$ mol$^{-1}$, both at pH 7 (Meggos, 1973). Stock solutions of fructose and glucose were prepared immediately before use, at a concentration of 40.01 g/100 ml in distilled, deionized water. A pH 7.16 sodium phosphate buffer was 1.0005 $M$ in phosphate; a Sargent-Welch Model PAX pH/Activity meter measured the pH of all solutions.

Complete decolorization of Red 2 was found to occur in unbuffered fructose solutions at 100°, although the pH dropped as low as 5.65. The final pH was found to be dependent on the original fructose concentration. Possible alterations in reaction mechanism with changes in pH necessitated the use of buffered solutions, even though phosphate and other common buffer anions reportedly enhance the rate of thermal degradation of reducing sugars (Hodge, 1953). Accordingly, quantitative data were obtained from reaction mixtures buffered with 0.114 $M$ phosphate at pH 7.16.

Reaction mixtures of 220 ml, containing $x$ milliliters of dye solution and $y$ milliliters of sugar solution, were prepared in a 300-ml three-necked flask fitted with reflux condenser and gas inlet tube. First, 25 ml of buffer, $x$ ml of dye, and $(195 - x - y)$ ml of distilled, deionized water were pipetted into the flask. At $t_0$ (zero time) $y$ ml of

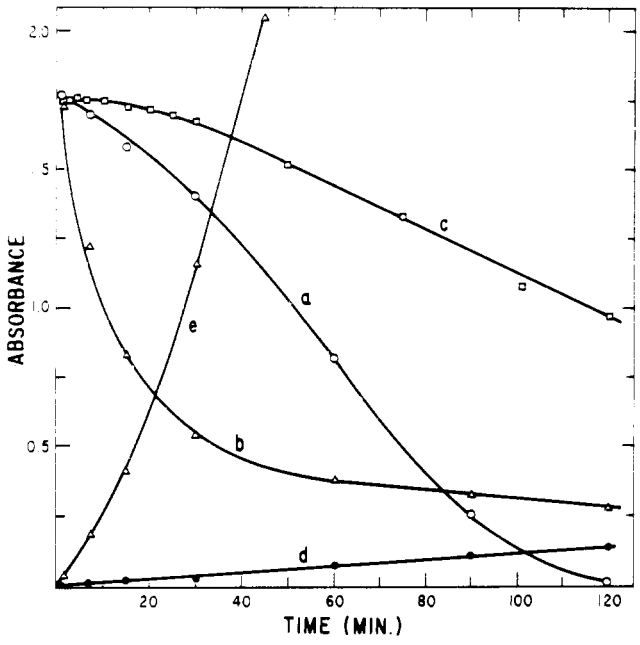


**Figure 1.** Absorbance changes in representative kinetics experiments at pH 7.16, 77°: (a) 0.101 *M* fructose, 6.73 × $10^{-5}$ *M* Red 2, under $N_2$; (b) 0.101 *M* fructose, 6.73 × $10^{-5}$ *M* Red 2, aerated; (c) 0.0505 *M* fructose, 6.73 × $10^{-5}$ *M* Red 2, under $N_2$; (d) 0.101 *M* fructose, under $N_2$; (e) 0.101 *M* fructose, aerated. Absorbance in experiments a, b, and c refers to $\lambda_{max}$ (Red 2) 522 nm; absorbance in d and e refers to 285 nm.

sugar solution was added and mixed thoroughly. In any set of experiments the maximum concentrations were such that $x = y = 10$ ml.

Constant temperature was maintained at 77.0 ± 0.5 and 61.0 ± 0.5° with a constant temperature water bath, and at 100° by refluxing on a heating mantle. The variation in boiling point over the encountered range of solute concentrations was calculated to be less than 0.05°, and in fact no change was observed. The buffered dye solution was equilibrated for at least 40 min at the appropriate temperature while purging with either Ultra High Purity $N_2$ or air at 200 ml/min flow rate. The solution temperature dropped briefly by as much as 2° upon the addition of the sugar aliquot, but recovered within less than 2 min.

Some replicate runs at 77 and 61° were conducted in 250-ml flasks without condensers. Nitrogen or air entered the solution through a glass tube inserted through a greased cork stopper. Dye solutions containing no sugar showed less than 3% evaporation loss at 77° over the maximum time period of these experiments.

Aliquots were withdrawn at appropriate times during the course of the reaction and, after immediate transfer to small screw-cap vials, cooled quickly in an ice bath. Ultraviolet and visible absorption spectra were recorded on a Beckman DK-2 spectrophotometer. Since the thermal degradation of reducing sugars produces chromophores with absorption throughout the visible spectrum (Liggett and Deitz, 1954; Ramaiah et al., 1956), reference solutions were generated by heating sugar solutions under identical conditions of concentration, pH, and atmosphere, taking aliquots at the same time intervals as the reaction runs. These reference solutions were also scanned in the uv and visible range against water to follow the rate of development of absorption maxima.

At 37.8°, the reacting solutions were tightly stoppered and placed in a constant temperature chamber. A very rough estimate of the time of half-reaction, $\tau_{1/2}$, was obtained by visually comparing the color intensity with that of an unheated solution in an identical container with dye concentration one-half that of the initial reacting solution.

In agreement with McCormick (1971), no reaction was observed at room temperature in 40 days. Aliquots were always returned to the reaction flask, followed by brief $N_2$ purging, in order to avoid losing all the solution before reaching $\tau_{1/2}$.

**Table I. Time of Half-Reaction of Azo Dyes with D-Fructose at 100°**

| Temp, °C | [Fructose]$_0$, *M* | $\tau_{1/2}$, min[a] | | |
|---|---|---|---|---|
| | | Red 2 ($N_2$) | Red 2 (air) | Red 40 ($N_2$) |
| 22.0 | 1.1 | N.R.[b] | N.R.[b] | N.R.[b] |
| 37.8 | 0.101 | 19 days | | |
| 61.0 | 0.101 | 93 ± 5 | | 135 ± 10 |
| | 0.0505 | 170 ± 12 | | |
| 77.0 | 0.101 | 60 ± 4 | 12 ± 4 | 38 ± 2 |
| | 0.0505 | 131 ± 8 | | 69 ± 4 |
| 100.0 | 0.101 | 54 ± 12 | 5 ± 2 | 31 ± 3 |
| | 0.0505 | 111 ± 10 | | |

[a] $\tau_{1/2}$ is independent of initial dye concentration, although these values refer specifically to 6.73 × $10^{-5}$ *M* (see Results and Discussion). [b] No decrease in absorbance at 522 nm (Red 2) or 505 nm (Red 40) in 40 days.

The experiments were discontinued at 40 days because the extensive growth of microorganisms hampered the measurement of the absorbance and changed the sugar concentration. The growth of microorganisms may also indicate that some oxygen had entered the sample containers.

Compounds listed at the beginning of this section are a few of the known products of reducing sugar degradation (Hodge, 1953; Shaw et al., 1968). Each was added to two portions of dye solution (6.73 × $10^{-5}$ *M* dye, pH 7.16) to give $10^{-3}$ *M* concentration, followed by anaerobic refluxing and heating at 77°, respectively. No attempt was made to accurately follow the kinetics of such reactions, other than the visible comparison method described above. The resultant solutions were saved for uv and visible absorption spectra.

## RESULTS AND DISCUSSION

**Reduction by Fructose.** The kinetic data for several representative experiments are presented in Figure 1. Clearly, the reaction of Red 2 with fructose initially proceeds much faster in an air atmosphere, as does the degradation of fructose because of direct air oxidation of the sugar (Fleming et al., 1968; Duborg and Naffa, 1959). The appearance of a pronounced induction period of longer duration than the time required for return to equilibrium temperature suggests that the dye reacts with one or more intermediate species formed by the thermal degradation of fructose. This induction period, as well as $\tau_{1/2}$, is considerably shorter in an oxygen environment for both Red 2 and Red 40 at each temperature studied. In some cases the temperature and concentration of fructose were sufficiently high that the induction period was scarcely observable.

The average values of $\tau_{1/2}$ for Red 2 and Red 40 decolorization in fructose solution at pH 7.16 are summarized in Table I. At constant temperature and initial fructose concentration, $\tau_{1/2}$ was found to be independent of initial dye concentration.

The experiments in air atmosphere had a greater variability in $\tau_{1/2}$ and induction time. Also, in those air atmosphere experiments where the concentration of fructose and the temperature were such that $\tau_{1/2} > 10$ min, the reaction apparently did not go to completion, i.e. the absorbance approached some limiting value. The spectra indicated that this infinite time absorbance might be attributable, at least in part, to the development of a new chromophore with absorbance centered at 490 nm.

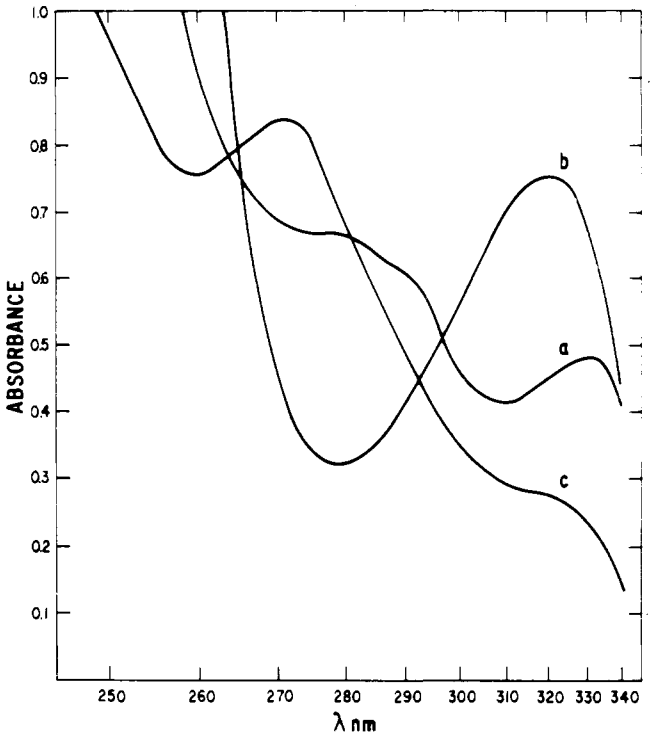


**Figure 2.** Ultraviolet spectra of Red 2 and its reduction products at pH 7: (a) Red 2, $4.32 \times 10^{-5}$ *M*; (b) initial reduction product by reaction with $Na_2SO_3$, tentatively identified as the hydrazo species, $4.32 \times 10^{-5}$ *M*; (c) reduction products of $SnCl_2$ reaction: two primary amines, $6.39 \times 10^{-6}$ *M* total concentration.

Other factors influencing the length of the induction period and $\tau_{1/2}$ were the age of the fructose stock solutions and the duration of preheating in several experiments in which dye was added to a sugar solution at $t_0$. In the first case, fructose stock solutions stored under refrigeration for 2 days prior to $t_0$ depressed $\tau_{1/2}$ by more than a factor of 2 and almost completely eliminated the induction period even under $N_2$. In the latter case, preheating the buffered fructose solution for 1 hr at 100° in air had little effect on $\tau_{1/2}$ but dramatically shortened the induction period.

**Reduction by Glucose.** At 100° under nitrogen, $\tau_{1/2}$ = 120 min for 0.101 *M* glucose–$6.73 \times 10^{-5}$ *M* Red 2. At 77° under nitrogen or air no apparent decrease in absorbance was detected in over 28 hr. On the contrary, absorbance rose slightly as a result of a small moisture loss by evaporation, since 250-ml stoppered flasks were used for these experiments. These same solutions, when stoppered tightly and heated for several more days at 77°, maintained their red color, deepened appreciably by the dark decomposition products of glucose. Evidently the induction period was still in progress or an alternative glucose degradation pathway predominated at this lower temperature.

Ultraviolet and visible spectra of glucose solutions at 77°, pH 7.16, showed a rapid development of chromophores at 267 and 345 nm, and most rapidly at 367 nm in air (slower development of the same chromophores under $N_2$), whereas at 100° the 267-nm chromophore increased much more rapidly than the other two and included a distinct shoulder at 280–285 nm. The higher temperature may be responsible for a faster and more complete degradation of glucose to yield most of the same products as fructose.

**Reduction Products of Red 2 and Red 40.** Both dyes were subjected to standard conditions for oxidation and reduction, i.e. $H_2O_2$, $Na_2SO_3$, and $SnCl_2$ in 5% HCl. The latter two reagents are reported to produce the hydrazo form and the completely reduced amine forms, respectively, in analogous azo dyes (Ogawa et al., 1971).

Attempts at oxidation with $H_2O_2$ (up to 7% aqueous solution, pH 1) failed, in that no change in spectrum was observed. The spectra of Red 2 and its reduction products are presented in Figure 2. Red 40 showed bands at 290 nm ($\epsilon = 1.53 \times 10^4$ l. cm$^{-1}$ mol$^{-1}$) and 248 nm ($\epsilon = 3.35 \times 10^4$ l. cm$^{-1}$ mol$^{-1}$) upon complete reduction by $SnCl_2$. Reduction of Red 40 by $Na_2SO_3$ produced a moderately intense band at 294 nm ($\epsilon = 2.22 \times 10^4$ l. cm$^{-1}$ mol$^{-1}$) plus an intense end absorption. In contrast, the normal azo form of Red 40 absorbs at 315, 268, and 235 nm. Extended reaction times of $Na_2SO_3$ reduction of both dyes resulted in spectra identical with those of $SnCl_2$ reduction.

Complete reduction by $SnCl_2$ is irreversible, as it involves scission of the -N=N- bond, but reduction to the hydrazo form is generally regarded as easily reversed by atmospheric oxygen (McKeown and Thomson, 1954). In contrast to expectations, partial reduction by sulfite was only incompletely, and slowly, reversible by atmospheric oxygen or even $H_2O_2$ in acidic solution. Solutions of Red 2 and Red 40 treated with $Na_2SO_3$ at neutral pH until achieving better than 90% color loss were then acidified with $H_2SO_4$ to liberate $SO_2$ from the excess sulfite. One-half of the resultant solution was purged with air, the other half was treated with 2–3% $H_2O_2$. Air was ineffective in reversing the reduction of either dye; peroxide effected only a 3–5% reversal, and then only very slowly.

Spectra of heated solutions of Red 2 plus fructose recorded vs. heated reference solutions of fructose showed the appearance of a distinct band at 320 nm with absorbance corresponding to a 75.8% conversion of the original Red 2 to the hydrazo species at completion. Evidence of a band at 272 nm was also apparent. Similar spectra of Red 40–fructose solutions showed broad, flat-topped peaks near 290 nm. The same absorption peaks were likewise observed in glucose reaction mixtures at 100°. Both dyes, therefore, seem to be reduced to a mixture of hydrazo and amine forms. The 320-nm band is not apparent in spectra of Red 40 reaction solutions and thus it is most probably not a product derived from either fructose or glucose, such as that obtained by reacting fructose with concentrated $H_2SO_4$ (Love, 1953). Furthermore, its appearance at reasonable intensity in reactions with fructose, glucose, and some of their derivatives (see below) seems to indicate its identity as the hydrazo species.

**Table II. Reduction of Red 2 by Sugar Degradation Products**

| Compound | Ca. $\tau_{1/2}$, min[a] | Appearance of 320-nm chromophore[b] |
|---|---|---|
| Acetaldehyde | N.R.[c] | |
| Acrolein (2-propenal) | N.R. | |
| Diacetyl (2,3-butanedione) | 300[d] | X[e] |
| Dihydroxyacetone | 50 | X |
| D-Glyceraldehyde | 20 | X |
| Glycolaldehyde | 30 | X |
| 3-Hydroxy-2-butanone | 90 | X |
| 5-Hydroxymethylfurfural (HMF) | 450[d] | X |
| Levulinic acid | N.R. | |
| Pyruvic acid | 60 | X[f] |
| Pyruvic aldehyde | 1200 | X |
| Triose reductone | 40 | |

[a] See Experimental Section for reaction conditions and techniques. [b] Tentatively identified as the hydrazo form of Red 2. [c] No reaction after more than 24-hr refluxing. [d] See Results and Discussion for method of estimation. [e] X = 320-nm chromophore observed in spectrum of final reaction solution. [f] 320 nm is approximately the $\lambda_{max}$ of pyruvic acid (Liggett and Deitz, 1954).

**Reduction by Sugar Degradation Products.** Table II lists the results of the reactions of Red 2 with sugar degradation products at 100°. Of those compounds which react rapidly with Red 2, only triose reductone completely reduces the dye to its corresponding amines. All degradation products had the same relative reactivities toward Red 40; $\tau_{1/2}$ values were roughly the same or slightly lower in every case. Those compounds which react at 100° also react at 77°. No $\tau_{1/2}$ is reported for diacetyl because the very rapid browning of diacetyl obscures the red color. During the course of refluxing, several aliquots were removed, filtered, and submitted to fourfold extraction in a separatory funnel with 50-ml portions of $Et_2O$ and then $CCl_4$, so that the final nonaqueous phase was colorless. Spectra of the aqueous fraction indicated that the normal azo form of the dye was no longer present after 2 days, but $\tau_{1/2}$ was longer than 5 hr. Similar problems were also encountered with HMF: along with a lengthy induction period for HMF browning is a concomitant induction period for Red 2 and Red 40 reduction, such that dark degradation products obscure $\tau_{1/2}$ observations. As with fructose and glucose solutions, none of the sugar degradation products caused fading of dye solutions at room temperature, even under $N_2$.

The rate of dye reduction by fructose follows the increase in absorbance at 285 nm of an equimolar fructose reference solution (Figure 1). Likewise, glucose solutions are reactive only after the appearance of a 285-nm chromophore. In all likelihood, the absorbance at 285 nm represents the sum of contributions from several chromophores, since most of the compounds listed in Table II have absorption maxima at or near that wavelength. This view is supported further by the observed shift of $\lambda_{max}$ to slightly longer wavelength as sugar degradation proceeds.

Since fructose undergoes many mechanistic pathways of degradation and rearrangement (Verstraeten, 1967), the reaction between Red 2 and fructose was studied under several other suitable conditions in order to find clues to the identity of the reactive intermediate species. First, Red 2 was added to a solution of thermally degraded fructose, pH 7.16 at room temperature, with no resultant reaction in 72 hr. Next, the highly reactive intermediates of fructose degradation were partially adsorbed on alumina, following the procedure of Song et al. (1966). The alumina was filtered off and equilibrated at room temperature with a solution of Red 2, again with no apparent reaction. These two experiments suggest that either the thermally produced reducing agents are not reactive at low temperatures or they are very unstable, perhaps with respect to air oxidation.

**Acidic pH.** Several anaerobic runs at 0.101 *M* fructose were made at 100°, 0.114 *M* $H_3PO_4$ instead of pH 7 phosphate buffer. A long induction period was encountered and $\tau_{1/2}$ was approximately 6 hr. An otherwise identical solution containing no dye was followed in the usual manner and the results were similar to those of Wolfrom et al. (1948): rapid appearance of chromophores at 230 and 285 nm which then convert to the spectrum of pure HMF. The concentration of HMF was calculated to equal $6.7 \times 10^{-5}$ *M* after 30 to 40 min. The observed rate of reaction with Red 2 at low pH would thus seem to be caused by some species in the HMF degradation pathway and not by HMF itself or fructose, particularly since Red 2 is less stable to reduction with decreasing pH (McKeown and Thomson, 1954).

**Alkaline pH.** Similar experiments were conducted in a 0.114 *M* phosphate buffer at pH 10.59. This time $\tau_{1/2}$ for 0.101 *M* fructose was slightly less than 5 min under $N_2$ at 100°. Again, fructose solutions showed rapid browning and development of the 285-nm chromophore, and even faster formation of a 295-nm chromophore, which may be the Berl and Feazel (1954) "sugar chromogen." At pH 10.59, the final solution of fructose and reduced Red 2 showed a 49.2% conversion to hydrazo, i.e. a larger percentage of the dye is completely reduced to amines at this higher pH.

**Effect of $Ca^{2+}$.** Fleming et al. (1968) refer to the inhibition of color formation and the preferential formation of saccharinic acids from fructose by treatment with $CaCl_2$. Therefore, the reaction between Red 2 and 0.101 *M* fructose was attempted in the presence of 2% $CaCl_2$. At pH 7.16, no color loss was observed in 72 hr of refluxing and a corresponding solution of fructose and $CaCl_2$ showed a slow conversion to HMF. As chloride ions have been implicated in the mechanism of the reaction between fructose and concentrated acid (Mendel et al., 1954), reactions were conducted using equivalent concentrations of calcium lactate or NaCl. Calcium lactate was also effective in inhibiting color formation from fructose and color fading of Red 2; NaCl had no apparent effect at neutral pH.

## SUMMARY

Heating of aqueous fructose and glucose produces strongly reducing substances which are capable of reducing azo dyes. Oxygen promotes the formation of these reducing agents, but it may also inhibit the reaction with azo dyes. The reduction of azo dyes can proceed before the accumulation of visibly colored sugar degradation products.

Fructose is more reactive than glucose under the conditions of this study. The reactivity of fructose is decreased by $Ca^{2+}$, and is highly pH dependent, in that the reaction proceeds much faster at high pH.

Several known sugar degradation products also reduce the azo dyes. Thus, the complex reaction mechanism implied by the shapes of the curves in Figure 1 probably involves several reactive intermediates in the degradation pathways of fructose and glucose.

## ACKNOWLEDGMENT

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