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A Browning Reaction Involving Copper-Proteins

J. B. Thompson,¹ R. B. Kocher² and H. W. Fritzsche

*From the Quartermaster Food and Container Institute
for the Armed Forces, Chicago, Illinois*

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INTRODUCTION

It is currently recognized that minute amounts of metals play important roles in biological reactions, both *in vivo* and *in vitro*. It has been established that many enzymes, and other biologically active proteins, contain metals as prosthetic groups and are more or less dependent upon these metals for their activity. Inasmuch as metallo-proteins have been shown to be significantly important biologically, a few examples are in order. Copper is found in living organisms as the prosthetic group of hemocyanin, polyphenol oxidase, hemocuprein and ascorbic acid oxidase; and iron is found in cytochrome oxidase, peroxidase and catalase (11). Copper and iron are not alone in representing metal constituents of enzymes. Manganese is a constituent of arginase, zinc of carboanhydrase, and aluminum of the complex succino-oxidase system in which it can be replaced by chromium (4). However, the mechanism of trace metal reactions is little understood. The literature is sketchy and, in many cases, contradictory.

In food chemistry it is a well recognized fact that off-flavors, oxidation and other deteriorations are often due to the presence of trace amounts of metals acting as catalysts. Since metals not only are present in foods in their natural state, but also may enter into the food as contaminants during compounding and processing, it is of commercial significance that a broader knowledge of their modes of action be obtained.

King (9) pointed out that copper may combine with certain proteins and react in analogous manner to ascorbic acid oxidase in the oxidation of ascorbic acid. It is possible that trace metal activity in food deterioration may be exerted mainly through reactions of this type. This

¹ Present address, Trace Metal Research Laboratory, 1665 East 79th Street, Chicago 49, Illinois.

² Present address, same as above.

paper will describe reactions of copper, proteins, and certain ring compounds containing an ethylene group. It will further be shown that complex end-products are formed which bear striking resemblance to some of the compounds involved in the browning reactions. In fact, it is now indicated that one type of non-enzymatic browning may be analogous to an enzymatic browning in that copper complexes with proteins, and the resulting copper-proteins act similarly to enzymes to which that metal is specific, but with deleterious effect.

Probably one of the more fundamentally sound approaches to the nature of the bonding of copper in biological materials has been made by Baudisch, who has found a simple reaction, called the B reaction, in which copper is almost unique in behaving as a central atom (2, 3, 5). This reaction is applicable to many aromatic hydrocarbons and many other ring compounds containing ethylene groups. It has also been used as a probable explanation for the activity of the copper in the polyphenol oxidases (5).

In a previous study of dry whole milk, an ideal medium for studying copper catalyzed deteriorations, it has been demonstrated that the copper is bound in the protein, that it is probably distributed uniformly in the major protein fractions, and that no electrovalent copper exists at normal pH values (15). Polarographic studies of casein containing copper indicate that the metal is strongly bound at pH 6.8-7.0.

Since work in this laboratory has strongly indicated that trace amounts of copper in foods are bound to proteins, an assumption was made that probably the mode of action might be similar to the activity of the enzyme to which the copper was specific. Assuming that the bonding of the copper might be similar to the bonding of the copper in the copper nitroso compound of the B reaction, it seemed reasonable that such copper proteins might react in an analogous manner to the cuprous salt of nitrosyl, and it was therefore decided to attempt to demonstrate complex formations between proteins which had been contaminated with copper and certain ring compounds containing ethylene groups.

EXPERIMENTAL

The first attempt was made by allowing a 1% solution of casein to react with an excess of *l*-ascorbic acid³ in the presence of a small amount

³ The concentration of *l*-ascorbic acid did not appear to be critical. In this experiment the weight ratio of ascorbic acid to casein was 1 to 10. However, the reaction can be demonstrated with much more or less ascorbic acid.

(0.1 g.) of finely divided copper oxide at 30°C. and pH 6.5-7.0. No attempt was made to protect the reaction from atmospheric oxygen or light. A distinct series of color changes occurred over a period of 24 hours. The color changed rapidly from white to yellow, and finally to a dark yellow. When the reaction time was increased, the color changes proceeded through orange to a brick-red at the end of 48 hours. Adjustment of the pH to 5.0 with HCl and treatment with 2-3 volumes of absolute ethanol precipitated a reddish-brown amorphous protein substance which could be taken up with dilute NaOH and reprecipitated with acid and ethanol. The substance possessed an isoelectric point which was almost identical with that of casein, was insoluble in water or dilute mineral acids, but was soluble in dilute NaOH. Analysis showed that it contained 110 mg./g. of N₂, 9 mg./g. of Cu, a fluorescence value equivalent to 0.3 γ of quinine sulfate/g., no reduced ascorbic acid as obtained by indophenol titration, and an indicated dehydroascorbic acid activity of 200 γ /g. as determined by the Roe method (12). It was also noted that the precipitate possessed a characteristic fragrant or fruity odor.

It was not possible to obtain a reaction if any one of the 3 reacting components (copper, casein, or ascorbic acid) was omitted. However, previous treatment of the protein solution with the copper oxide, followed by filtration of the excess oxide and then adding the ascorbic acid resulted in the typical reaction. Thus, it seemed reasonably well established that the copper was bound with the protein and that the resulting copper-protein reacted with the ascorbic acid. The ability of a 1% casein solution to readily pick up copper from copper oxide at normal pH and temperature is in itself significant, in that it throws light on the ease with which foods pick up copper during processing in copper equipment.

The investigation was broadened to include the reacting of copper and casein with several compounds containing an ethylene group within a ring structure as well as certain other compounds not containing this group. These materials are listed in Table I as "reactive" or "non-reactive." It is interesting to note that compounds containing

$$\begin{array}{c} | \quad | \\ -C=C- \end{array}$$

groups within a ring gave similar reactions to that described for ascorbic acid, while glucose and lactose were unreactive. Glucose, in particular, is known for its role in browning through its reaction with amino acids. This strongly indicates that the browning occurring

TABLE I

Materials Investigated for Reactivity with Copper-Proteins
(pH 6.5-7.0)

Reactive	Non-Reactive
<i>l</i> -Ascorbic acid	Oxalic acid
Reductic acid ^a	Glucose
Furfural	Lactose
Furfuryl alcohol	Pectin
Catechol	
Hydroquinone	
Phenol	
Pectin, acid hydrolyzed	

^a The fluorescing properties of the browning of reductic acid are now under investigation and will be reported by Dr. T. E. Friedemann, Passavant Memorial Hospital, Chicago; trace metal activity does not come within the scope of the investigation.

with copper-casein is not the typical browning due to amine-aldehyde interaction, but may be due to a complex formation between the copper-casein and the reacting substance, in which the copper possibly acts as the central atom. The reactivity of the hydrolyzed pectin can be explained on the basis of molecular changes during the hydrolysis.

In 24 experiments reacting copper-casein with the 8 reacting substances in Table I, it was noted that there resulted no consistent or reproducible concentration of copper in the complex end-products, the copper ranging from 0.5 to 14.0 mg./g. This can undoubtedly be attributed to the fact that the protein binds copper in excess of that necessary to produce the reaction. The nitrogen contents were fairly uniform, ranging between 90 and 120 mg./g., and there was no correlation between the nitrogen and the copper contents.

All of the complex end-products formed were studied for their fluorescing properties, and polarograms from 0 to -3 volts were made on each.

Samples were dissolved in dilute NaOH and adjusted to pH 6.0 with HCl. Fluorescence values were determined on a saline solution (10), while the polarograms were made directly on the filtered solution. The fluorescence values were remarkably reproducible (within 15%) for any one of the reacting substances in Table I, but there were significant differences in fluorescence values of different reacting substances. The polarograms demonstrated two half-wave potentials which were common to all of the substances, including the unreacted copper-casein. The half-wave potentials were at -0.25 and -1.2 volts. There was a significant difference, however, for different reacting substances. For example, the ascorbic acid reaction end-product

exhibited strong waves at both half-wave potentials, while less reactive substances exhibited fairly strong waves at -0.25 volts and small waves at -1.2 volts. The unreacted copper-casein showed a fairly strong wave at -0.25 volts and a negligible wave at -1.2 volts.

To establish the order of reactivity of the 8 reacting substances with copper-casein, a controlled experiment was made in which a large amount of casein was contaminated with copper to the extent of 14 mg./g. of casein. In this particular case ionic copper was used in place of the oxide and the resulting copper-casein was blue.

TABLE II

Reactivity of Copper-Casein with Compounds Containing
an Ethylene Group in a Ring Structure

Substance added to Cu-casein	Color			Reactivity		
	During reaction		End-product	Based on color change	Based on fluorescence	Based on step height ratio
	0.5 hr.	24 hrs.	(Dry)	Visual observations	γ of quinine sulfate/g.	$\frac{I_{-0.25V}}{I_{-1.2V}}$
Reductic acid	Orange-yellow	Dark gray	Brown-black	++++	0.69	1.3
<i>l</i> -Ascorbic acid	Light yellow	Dark yellow	Brown	+++	0.28	1.4
Catechol	Red-brown	Red-black	Red-black	++++	0.22	1.4
Hydroquinone	Dark gray	Red-black	Red-brown	+++	0.20	1.3
Furfural	Yellow-orange	Orange-brown	Red-brown	++	0.19	1.2
Furfuryl alcohol	Yellow-orange	Orange-brown	Yellow-brown	++	0.15	1.3
Pectin, hydrolyzed	Blue	Blue	Dark blue	+	0.10	1.7
Phenol	Blue	Gray-blue	Gray-blue	+	0.05	2.2
Blank	Blue	Blue	Blue	0	Trace	2.0

(Copper oxide and copper ion can be used interchangeably with the same general results, except that the color of the casein becomes blue when sufficient of the ionic copper is used.) Identical volumes of the 1% solution were treated with the various reacting materials for exactly 24 hours at 30°C. at pH 6.6–6.8. The results of the test are presented in Table II, in which it is demonstrated that there is a definite order of reactivity of the various reacting substances as well as a correlation between the visual observations of color changes, the degree of fluorescence, and the ratio of the step height at -0.25 volts to that at -1.2 volts. Since the concentration of a given component at a given half-wave potential is a function of the current, the expression for the step height ratio is conveniently stated as $I_{-0.25V}/I_{-1.2V}$.

The blue color of the copper-casein disappeared in order of increasing activity. Thus, the blank consisting of copper-casein alone retains its blue color, while phenol and hydrolyzed pectin darken, and in all others the blue color of the cupric ion disappeared immediately and prior to any darkening. Both phenol and hydrolyzed pectin will continue to react if given a longer period of time, in which case they also yield a dark brown end-product free of the blue color with an $I_{-0.25V}/I_{-1.2V}$ of about 1.2 and 1.3. It appears that when the maximum fluorescence has developed for a given reaction the $I_{-0.25V}/I_{-1.2V}$ will be 1.2–1.4, and that a higher value indicates incomplete reactions. Although much remains to be investigated on the fluorescence and polarographic approaches, the indicated correlation between the two is certainly noteworthy.

Having established the general activity of the conjugated protein, casein, it was decided to investigate a simple protein such as albumin. It was found that, like casein, albumin entered into generally similar

reactions with copper and compounds containing a $\begin{array}{c} | \\ -C=C- \\ | \end{array}$ group in a ring structure, yielding similar end products, but was more selective than casein. For example, copper-albumin reacted very rapidly with the aromatic compounds, but required several days to react with ascorbic acid, and then produced only small amounts of the complex end-product. Gelatin reacted similarly to albumin. Table III shows the proteins and amino acids studied and lists them as "reactive" and "unreactive."

The question of how the copper is bound in the protein still remained. If the copper were bound to free polar groups, it should be possible to replace the protein with amino acids in the reactions. A complete acid hydrolyzate of casein was selected as an ideal substitute. Neither the hydrolyzate nor the two amino acids, tryptophan and cystine in which

TABLE III
Proteins and Amino Acids Investigated
(pH 6.5–7.0)

Reactive	Non-reactive
Casein ^a	Casein hydrolyzate
Albumin ^b	(complete acid)
Gelatin	Tryptophan
Bacto-peptone	Cystine

^a Prepared from dry milk by sodium chloride precipitation (13, 14, 15), washed repeatedly.

^b Prepared from fresh eggs by separation, washing and filtration (6).

the hydrolyzate was deficient, were capable of replacing casein in the reactions. Thus, the postulate that the copper was bound through forces existent in polypeptides and not to amino acids was given strong support. Substitution of Bacto-peptone for protein gave analogous reactions to albumin, but this would be anticipated since peptones contain an abundance of peptides.

DISCUSSION

It seems reasonable to suggest that the reaction mechanism involved in this investigation is one in which trace amounts of copper rapidly bind to polypeptides and the resulting copper-protein combines with certain ring compounds containing ethylene groups. The possibility of such types of reactions occurring in foods or, for that matter, other biological materials which contain copper, either naturally or through contamination, is obvious on the basis of the composition of those materials. It is further suggested that trace amounts of copper bound to proteins react in an analogous manner to the enzymes to which copper is specific. Considering the specificity of such enzymes as ascorbic acid oxidase or tyrosinase, it becomes obvious that specific types of linkage are involved. On the other hand, when whole proteins are contaminated with copper the copper distribution is undoubtedly quite general. Thus, with copper-casein we might expect the quite generalized reactions obtained as contrasted to the specificity of the enzymes. It does seem reasonable, however, that different proteins would demonstrate some generalized specificity for certain groups of compounds on the basis of the difference in protein molecular structure. Thus, it is not surprising that we find copper-casein generally reactant to all the compounds

investigated, while copper-albumin is more selective. The latter reacted immediately with the aromatic compounds, but very slowly with ascorbic acid.

It is well recognized that the browning of foods is extremely complex and involves many reactions. Although it has been suspected that trace metals may catalyze browning, it was only recently that it was shown that trace amounts of metals, particularly copper, materially accelerate the rate of browning (1). The results of the present investigation show that there is probably a very marked involvement of, at least, copper.⁴ The complex end-products which have been isolated all possess common characteristics of browning end-products. They range in color from dark reddish-brown to black, possess a high degree of fluorescence, and are characterized by odors which can generally be classified as fragrant, acid, burnt and caprylic in accordance to the Crocker-Henderson Test (7, 8). The complexity of the odors was well evidenced by the remarks of several testers when they examined these materials. For one of the samples they suggested "fragrant," "vanilla-like," "hint of garlic," "burnt," and "fruity." In the authors' opinion "fruity" and "caramel" odors predominated in all of the compounds except those obtained using furfural and furfuryl alcohol, which retained their typical furfural odor.

It is significant that these reactions were obtained at near room temperatures (about 30°C.) and normal pH. Although no special effort was made to study the reaction at elevated temperatures, it was noted in a few tests with copper-albumin and *l*-ascorbic acid that the reaction could be accelerated by elevating the temperature to 50°C.

Results of this investigation have indicated that trace amounts of copper in foods are bound to proteins or polypeptides and can result in a browning reaction which appears to be very similar to certain types of enzymatic browning. Since other metals may exert their activity in a somewhat analogous manner to copper, the significance of this type of reaction cannot be overlooked. It is necessary that enzymes must be inactivated if a processed food is to have maximum stability. However, the blanching employed to accomplish inactivation does not decrease the concentration of the metals present, and it may even increase the metal content through contamination during the process. It is possible that, in certain cases, recurrence of enzymatic activity,

⁴ In naturally occurring systems the copper concentrations would be much lower than those studied in this investigation. However, it may be presumed that the diluted reactions would proceed at a slower rate.

which is usually attributed to incomplete or inadequate blanching, may in actuality be due to the presence of one or more metals in the form of metallo-proteins.

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SUMMARY

1. Reactions of copper, proteins and certain ring compounds containing an ethylene group are described. These reactions are accompanied by marked changes in color as well as development of fragrant odors and fluorescence.

2. It is shown that proteins can readily bind copper from copper oxide, and that the copper in the active complex is probably bound through forces existent in polypeptides and not to amino acids.

3. It is indicated that copper-protein complexes can promote a browning, the mechanism of which is different from known types of non-enzymatic browning.

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