the denaturing conditions (Figures 4-9 and Table II). The condition of myosin A in the fiber can be judged by investigating the loss of extractability.

Although the rate of thermal denaturation of myosin A in the glycerol-treated fiber bundles was considerably slower than that observed in the isolated myosin A (Figures 6-8), it seemed to be more sensitive to temperature than to pH (Figures 5-8). It is a well-known fact that pale watery meat occurs when the rate of postmortem pH fall is three or more times higher than the normal rate and the muscle is not allowed to cool below 38° before the pH falls below 6.0 (Briskey and Wismer-Pedersen, 1961; Wismer-Pedersen, 1959). The results mentioned above will give an important clue in explaining the change in the quality of pale watery meat.

The greater stability of myosin A in the muscle over the isolated molecules was not fully determined. It is, however, very likely that, in muscle undergoing rigor in conditions of high temperature and low pH, the highly organized contractile proteins of myofibrils could be more resistant to environmental changes due to the restriction of thermal motion of the molecules. Fusion of molecules which caused aggregation and masking of active sites observed on myosin systems (Kawakami *et al.,* 1971) would be expected to take place locally in the rigid built-in system (Huxley, 1963; Reedy *et al.,* 1965) such as glycerol-treated fiber bundles. This expectation was borne out by the results in Figures 6-8, as well as Figure 9 and Table II, which show only that the native form of myosin A was extracted, in spite of the total decrease in extractability.

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# Model Studies Regarding the Internal Corrosion of Tin-Plated Food Cans. III. On the Binding of Tin(II) Ions and Iron(II) Ions by Sulfur-Containing Amino Acids

Dieter W. Gruenwedel* and Hsien-Chung Hao

Stability constants of chelates formed by tin(II) and iron(II) ions with *l*-cysteine hydrochloride and *dl*-penicillamine were determined by pH titrations at 25° and in 0.1 *M* KCl. With Fe(II), cysteine, as well as penicillamine, forms stable complexes of the type ML and $ML_2$ at pH values above 6. With Sn(II), the ligands form only ML chelates; however, complex formation proceeds already at pH values above 2. Indications are that in all instances hydroxo complexes are formed at high pH values. However, these reactions were not investigated in any detail due to the onset of precipitation. The results are discussed in relation to the well-known reversal in polarity of the tin-iron couple observed in cans containing low-acid, protein-rich food materials.

It is generally believed that the protection offered by tin to steel at the interior surface of tin-plated food cans is a sacrificial one. Contrary to its position in the electrochemical series of the elements, which would require tin to be cathodic to iron, the metal becomes the anode in the local electrolytic cells that are set up when tin plate is in contact with the liquid medium of the food product. In such instances the protective coating (tin) will undergo slow even corrosion, whereas the base metal (iron), exposed under the pores or cracks that exist in the coating, acts as the local cathode (Hoar, 1934; Kohman and Sanborn, 1928a,b,c; Lueck and Blair, 1928a,b; Mantell and King, 1927; Mantell and Lincoln, 1926).

While there has been no exact agreement as to the cause of the observed change in polarity (*cf.* Hartwell, 1951; Hoare *et al.,* 1965; Koehler, 1961) it appears that one major contributing factor is the ability of the liquid medium to bind free stannous ions (Hoar, 1934). From simple thermodynamic considerations alone it can be readily shown that the electrode potential of tin has to become more negative than that of iron as soon as the ratio of the concentration of stannous ions to ferrous ions, $[Sn^{2+}]/[Fe^{2+}]$, is smaller than the value $5 \times 10^{-11}$, *i.e.,* free Sn(II) ions are present only in extremely minute amounts. This is of course a consequence of the concen-

Department of Food Science and Technology, University of California, Davis, California 95616.

tration dependence of the electromotive force of reversible electrodes (Nernst equation).

It is clear that a concentration ratio $[Sn^{2+}]/[Fe^{2+}] \leqq 5 \times 10^{-11}$ can occur only through the formation of very stable tin complexes. In regards to high-acid food products such as tomatoes, rhubarb, or fruit juices, it is known that the organic acids they contain bind Sn(II) ions very strongly. For instance, citric acid and tartaric acid form highly stable Sn(II) chelates, particularly at the low pH values between 3.5–4 which are commonly encountered in these foods (Smith, 1965). By contrast, Fe(II) ions are chelated only very weakly under the same pH conditions (Hamm *et al.*, 1954). These findings thus are in agreement with the concept of the reversal in polarity due to complex formation.

Our knowledge concerning the interdependence of polarity and complex stability in the case of low-acid, protein-rich food products is less detailed. It appears that in canned seafood, tin is also anodic to iron. This was shown by Pigott and Dollar (1963) who studied the polarity of the tin–iron couple in tuna packs and salmon packs as a function of temperature. Of particular interest hereby is their observation that tin acts as the local anode as long as it is in contact with the food material, whereas iron will be anodic to tin when the tin plate is separated from the food; *e.g.*, as this is the case in the headspace area. Surprisingly enough, however, virtually nothing is known about the interaction of the proteins and amino acids which these foods contain with Sn(II) ions, and information concerning their reactivity with Fe(II) ions is also not abundant. In part, this is undoubtedly due to the quite complex chemistry of aqueous solutions of Sn(II) (Tobias, 1958). On the other hand, it may also have been due to the fact that tin does not appear to be of any significance in biochemical reactions. Thus, the scarcity of data regarding the interaction of some of the major constituents of protein-rich foods with tin ions made it seem worthwhile to embark upon this investigation. In this work we have used the amino acids *l*-cysteine and *dl*-penicillamine as representative examples of the protein constituents of low-acid foods in order to see whether or not the polarity observed in canned seafood is in agreement with existing chelate stability. Furthermore, since cysteine in particular has been implicated in certain internal can corrosion processes such as the so-called "sulfide blackening" of meat products (Johnson and Vickery, 1964) and seafood products (Gruenwedel and Chu, 1972; Gruenwedel and Patnaik, 1971; Pigott and Dollar, 1963), a knowledge of its avidity for Sn(II) and Fe(II) might prove helpful in understanding more clearly the nature of the discoloration reaction.

This communication describes the results of our potentiometric pH titration studies. In a subsequent paper we will report about a further characterization of the Sn(II) complexes of cysteine using a variety of voltammetric techniques (Gruenwedel and Patnaik, 1972a).

## EXPERIMENTAL SECTION

**Materials.** *l*-Cysteine hydrochloride monohydrate and *dl*-penicillamine were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Potentiometric standardization with KOH showed that they were of excellent quality, and they were therefore used without further purification. Reagent grade $FeSO_4 \cdot 7H_2O$ and $SnCl_2 \cdot 2H_2O$ were obtained from Mallinckrodt. In view of the ease with which Fe(II)- and Sn(II) solutions are oxidized in the presence of air to Fe(III) and Sn(IV), respectively, they were prepared anew each time just prior to use. Fe(II) solutions were standardized with disodium ethylenediaminetetraacetate (EDTA) using variamine blue as an indicator (Schwarzenbach and Flaschka, 1965). Sn(II) concentrations were determined potentiometrically by using standardized KOH. Distilled and subsequently doubly deionized water was used throughout the investigation. It was boiled for at least 15 min prior to use to expel oxygen and cooled to room temperature while passing oxygen-free nitrogen through it.

**Methods.** *Measurements.* Potentiometric titrations were performed using the Orion Model 801 digital pH meter (Orion Research, Inc., Cambridge, Mass.). The pH of the solutions was monitored with a Metrohm Model EA125U semimicro combination glass electrode (Metrohm Division, Brinkmann Instruments, Inc., Westbury, N. Y.). The combination glass electrode, together with the solution under investigation, formed the electrochemical cell: glass electrode/solution, pH ($\mu$ = 0.1 *M* KCl)/4.0 *M* KCl (saturated with AgCl)/AgCl/Ag. The Nernst slope of this cell was frequently determined with the help of the slope calibrator of the Model 801 digital pH meter using a variety of commercially available pH buffers as pH standards. The cell exhibited the theoretical Nernst slope within the pH range 4–9, whereas the slope decreased to 98.5% of the theoretical value above and below this range, *e.g.*, within the pH regions 2–4 and 9–12. Thus, during the course of a titration, the pH values measured within the latter two regions were corrected for deviation from linearity by appropriate changes in the calibrator setting. Otherwise, for quick glass electrode standardizations, the buffers pH 4.00 and pH 7.00 (25°) (Matheson, Coleman, and Bell) were used.

The ionic strength, $\mu$, of the experimental solutions was maintained at 0.1 *M* by adding calculated quantities of potassium chloride. All measurements were performed in a thermostated titration vessel at 25 ± 0.02° and in an atmosphere of nitrogen. Stirring was accomplished with the help of a magnetic stirring bar. Ligand solutions were prepared by dissolving the amino acid hydrochlorides in water; penicillamine was converted into the hydrochloride by adding a known amount of hydrochloric acid to its solution. The normality of each ligand solution was determined by titration with standardized KOH.

The proton association constants of the amino acids were determined by titration of the fully protonated ligands with standardized KOH in the absence of the complexing metal ions. KOH was added to the ligand solutions with the help of a Gilmont Ultra-Precision Micrometer buret which has a delivery accuracy of 0.02%.

The formation constants of the metal chelates were determined by titration of the fully protonated ligands with KOH in the presence of the complexing metal ions.

*Calculations.* The experimental data were the four parameters: $L_t$, the total amino acid (ligand) concentration; $M_t$, the total complex-forming metal ion concentration; $H_t$, *the total hydrogen ion concentration in the solution* under investigation; and [H], the equilibrium hydrogen ion concentration in the system defined by

$$pH_{measured} = -\log[H] \tag{1}$$

The triad $L_t$, $M_t$, and $H_t$ can be expressed in terms of the equilibrium concentrations of the various species which participate in complex formation as follows.

$$L_t = \Sigma_{n=0}[H_nL] + \Sigma_{i=0} i[ML_i] \tag{2}$$

$$M_t = \Sigma_{i=0}[ML_i] + \Sigma_{q=1}\Sigma_{r=1} q[M_q(OH)_r] \tag{3}$$

$$H_t = [H] - [OH] + \Sigma_{n=0} n[H_nL] - \Sigma_{q=1}\Sigma_{r=1} r[M_q(OH)_r] \tag{4}$$

In eq 2–4, $\Sigma_{n=0}[H_nL]$ is the concentration of any uncom-

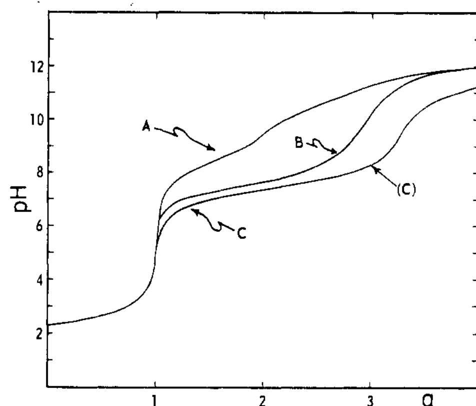



**Figure 1.** pH titration of *l*-cysteine hydrochloride with potassium hydroxide: curve A, free ligand; curve B, ligand in the presence of Fe(II) ($L_t:M_t$ = 2:1); curve C, ligand in the presence of an equimolar concentration of Fe(II). Concentration of ligand $L_t$ = 0.01 *M*. Ionic strength $\mu$ = 0.1 *M* (KCl); *a* = added equivalents of potassium hydroxide. The straight arrow C indicates onset of precipitation.

plexed amino acid composed of several differently protonated species; *i.e.*, [L], for instance, stands for the cysteinate anion. $\Sigma_{i=0} i[ML_i]$ is the concentration of a complexed amino acid and $\Sigma_{n=0} n[H_nL]$ is the concentration of the hydrogen ions which are bound to an uncomplexed amino acid. $\Sigma_{i=1}[ML_i]$ is the concentration of a complexed metal ion, whereas $[ML_i]_{i=0}$ = [M] is the concentration of a free metal ion. [H] and [OH] are the concentrations of the free hydrogen ions and free hydroxide ions in the experimental systems whereby [OH] can be easily calculated from the measured pH with the help of the known ion product $K_w$ of water. We used the value $K_w = 1.631 \times 10^{-14}$ (mol²/l.²) (25°, $\mu$ = 0.1 *M* KCl) in this work (Harned and Hamer, 1933). The expressions $\Sigma_{q=1}\Sigma_{r=1} q[M_q(OH)_r]$ and $\Sigma_{q=1}\Sigma_{r=1} r[M_q(OH)_r]$ take into account that the complex-forming metal ions can potentially also interact with the solvent; *e.g.*,

$$qM + rH_2O \rightleftharpoons M_q(OH)_r + rH \quad (5)$$

The two expressions represent, respectively, the concentration of the various metal hydroxo complexes formed and the concentration of the hydrogen ions liberated in the system due to hydroxo complex formation. All ionic charges have been omitted from the above equations for reasons of clarity and they also will not be presented elsewhere in this paper.

If we define with

$$\beta_n^{\ H} = [H_nL]/[H]^n[L] \ (\beta_0^{\ H} = 1) \quad (6)$$

$$\beta_i^{\ M} = [ML_i]/[M][L]^i (\beta_0^{\ M} = 1) \quad (7)$$

$$\beta_{q,r}^{\ MOH} = [M_q(OH)_r][H]^r/[M]^q (\beta_{1,0}^{\ MOH} = 1 \text{ and } \beta_{0,1}^{\ MOH} = (K_w)^r) \quad (8)$$

where $\beta_n^{\ H}$, $\beta_i^{\ M}$, and $\beta_{q,r}^{\ MOH}$ are, respectively, the overall proton association constant of the amino acids, the overall complex formation constant of the metal chelates, and the overall metal hydroxide formation constant, eq 2–4 can be also expressed in terms of eq 6–8; *e.g.*,

$$L_t = \Sigma_{n=0} \beta_n^{\ H}[H]^n[L] + \Sigma_{i=0} i\beta_i^{\ M}[M][L]^i \quad (9)$$

$$M_t = \Sigma_{i=0} \beta_i^{\ M}[M][L]^i + \Sigma_{q=1}\Sigma_{r=1} \beta_{q,r}^{\ MOH} q[M]^q[H]^{-r} \quad (10)$$

$$H_t = [H] - [OH] + \Sigma_{n=0} n\beta_n^{\ H}[H]^n[L] - \Sigma_{q=1}\Sigma_{r=1} r\beta_{q,r}^{\ MOH}[M]^q[H]^{-r} \quad (11)$$

Equations 9–11 were used to calculate the various constants with the help of the known experimental parameters $L_t$, $M_t$, $H_t$, [H], and [OH]. Obviously, $H_t$ is given by the general relation

$$H_t = NL_t - K_t \quad (12)$$

where $N$ is the maximum number of hydrogen ions associated with the ligand at the beginning of a titration and $K_t$ is the concentration of added titrant, *i.e.*, KOH.

The calculations were performed using well-known algebraic methods (Rossotti and Rossotti, 1961; Schläfer, 1961; Schwarzenbach, 1950). The proton association constants of the amino acids were calculated with an Olivetti Underwood Programma 101 electronic desk calculator. The metal complex formation constants were obtained by employing the Burrough 5500 Computer of the Computer Center of the University of California, Davis, Calif. The computer programs were written in Fortran IV language.

## RESULTS AND DISCUSSION

**Metal Binding of the Ligands—Interpretation of the Titration Curves.** Figure 1, curve A, displays the variation of pH in a solution containing only *l*-cysteine hydrochloride when KOH is added. Based on our knowledge of the acid–base properties of amino acids, the buffer region stretching from $a$ = 0 to $a$ = 1 ($a = K_t/L_t \equiv$ added base equivalents) corresponds to the dissociation of the $\alpha$-carboxyl group of cysteine, whereas the protonated $\alpha$-amino group releases its hydrogen ion between $a$ = 1 and $a$ = 2. The HS group of *l*-cysteine becomes ionized in the buffer region $a$ = 2 to $a$ = 3. In the case of curve A, $M_t$ = 0, and eq 9 and 11 can be greatly simplified. With $n$ assuming sequentially the values 0, 1, 2, and 3 and, further, by setting $(H_t - [H] + [OH])/L_t \equiv n_H$, a combination of eq 9 and 11 yields

$$\Sigma^3_{n=0}(n_H - n)\beta_n^{\ H}[H]^n = 0 \quad (13)$$

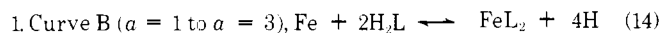
from which the various $\beta_n^{\ H}$'s are easily evaluated (*cf.*, Schwarzenbach, 1950). $n_H$ is defined as the average number of hydrogen ions attached to one ligand molecule. Obviously, $n_H$ can be calculated for each point of curve A from the known parameters $H_t$, $L_t$, [H], and [OH]. $H_t = 3L_t - K_t$ in the case of cysteine hydrochloride (*cf.*, eq 12).

It can be readily shown that $\log \beta_1^{\ H} = pK_{SH}$, $\log(\beta_2^{\ H}/\beta_1^{\ H}) = pK_{NH_2}$, and $\log(\beta_3^{\ H}/\beta_2^{\ H}) = pK_{COOH}$ of *l*-cysteine, where p*K* here has its usual meaning; *i.e.*, it is the negative logarithm of the dissociation constant of the hydrogen ion binding site under consideration. The various p*K*'s of *l*-cysteine, together with those of *dl*-penicillamine (*cf.*, Figure 2, curve A), are listed in Table I. The constants were determined from several individual titration curves. They are in excellent agreement with published data (*cf.*, Albert, 1952; Doornbos and Faber, 1964).

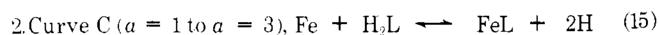
**Table I. Dissociation Constants of *l*-Cysteine and *dl*-Penicillamine ($\mu$ = 0.1*M* KCl, *t* = 25°)**

| | p*K* | | |
|---|---|---|---|
| Ligand | $\alpha$-COOH | $\alpha$-NH$_2$ | -SH |
| HSCH$_2$CH-(NH$_2$)COOH (cysteine) | 2.21 ± 0.02 | 8.32 ± 0.04 | 10.57 ± 0.05 |
| HSC(CH$_3$)$_2$-CH(NH$_2$)COOH (penicillamine) | 2.20 ± 0.05 | 8.09 ± 0.02 | 10.68 ± 0.09 |

Curves B and C, Figure 1, show that Fe(II) is bound by $l$-cysteine according to:



1. Curve B ($a$ = 1 to $a$ = 3), $Fe + 2H_2L \longrightarrow FeL_2 + 4H$ (14)



2. Curve C ($a$ = 1 to $a$ = 3), $Fe + H_2L \longrightarrow FeL + 2H$ (15)

In the case of curve C, Fe(II)-cysteinato 1:1 chelate, there seems to be an additional reaction involving the formation of hydroxo complexes; *e.g.*, according to $FeL + H_2O \rightarrow Fe(OH)L + H$. This is indicated by the fact that at pH values above 9 and $a > 3$, the neutralization curve C does not coincide with the neutralization curve A of the metal-free ligand. Tanaka *et al.* (1955) report for the reaction $Fe(II) + OH + L \rightarrow Fe(OH)L$, where L stands for the cysteinate anion, an equilibrium constant of log K = 12.77. However, we did not investigate this part of the reaction due to onset of precipitation in our system.

Reactions 14 and 15 can be readily evaluated in the buffer regions stretching from $a$ = 1 to $a$ = 3. We set $r$ = 0 and $q$ = 0 (*cf.*, eq 10 and 11) since Fe(II) does not interact with water according to reaction 5 in the presence of cysteine. Further, by defining with $n_L$ the average number of ligands bound per metal ion, *e.g.*,

$$n_L = (L_t - [L]\Sigma^3_{n=0}\beta_n^H[H]^n)/M_t \quad (16)$$

a combination of eq 9 and 10 gives

$$\Sigma^2_{i=0}(n_L - i)\beta_i^{Fe}[L]^i = 0 \quad (17)$$

from which $\beta_1^{Fe}$ and $\beta_2^{Fe}$ can be determined once $n_L$ and [L] are known. We calculate values of [L] from eq 11 since $H_t$, [H], [OH], $\beta_1^H$, $\beta_2^H$, and $\beta_3^H$ are known for each point of the titration curves B and C. By inserting [L] into eq 16, $n_L$ is obtained. In the case of curve C (Figure 1), the experimental conditions are such that $M_t = L_t$. This means that $0 \le i \le 1$, and eq 17 reduces to the simple expression $\beta_1^{Fe} = n_L/(1 - n_L)[L]$. In curve B, $L_t = 2M_t$; *i.e.*, $0 \le i \le 2$. Thus, the complete eq 17 is used. Table II contains the values of $\beta_1^{Fe}$ and $\beta_2^{Fe}$ for $l$-cysteine as well as $dl$-penicillamine. Obviously, the ratio $\beta_2^{Fe}/\beta_1^{Fe} \equiv [FeL_2]/[FeL][L] = K_2^{Fe}$ represents the so-called step stability constant of the 1:2 chelate. It describes the equilibrium $FeL + L \rightarrow FeL_2$. We have listed $\beta_2^{Fe}/\beta_1^{Fe}$ in Table II also. In general, the chelate formation constants vary by about ±0.03 log-units. The constants of the Fe(II)-chelates are in good agreement with published data (*cf.*, Doornbos and Faber, 1964; Sillén and Mautell, 1964).

The neutralization curves obtained by titrating $dl$-penicillamine with alkali in the presence of Fe(II) are very similar to those obtained with cysteine. We therefore did not include them in this publication.

In Figure 2, curve B represents the neutralization curve of Sn(II) in the absence of any complex-forming ligand. An excess of HCl, corresponding to half a base equivalent, had been added to the solution prior to titration in order to prevent the immediate hydrolysis of Sn(II). Thus, from

**Table II. Chelate Formation Constants of $l$-Cysteine and $dl$-Penicillamine ($\mu$ = 0.1 *M* KCl, $t$ = 25°)**

| Ligand | log $\beta_1^{Fe}$ | log $\beta_2^{Fe}$ | log $\frac{[FeL_2]}{[FeL][L]}$ | log $\beta_1^{Sn}$ [a] | log $\beta_1^{Sn}$ [b] |
|---|---|---|---|---|---|
| Cysteine | 6.78 | 11.49 | 4.71 | 15.20 | 15.15 |
| Penicillamine | 7.19 | 13.55 | 6.36 | 16.33 | 16.36 |

[a] Calculated according to eq 9–11. [b] Calculated according to eq 16 and 17.

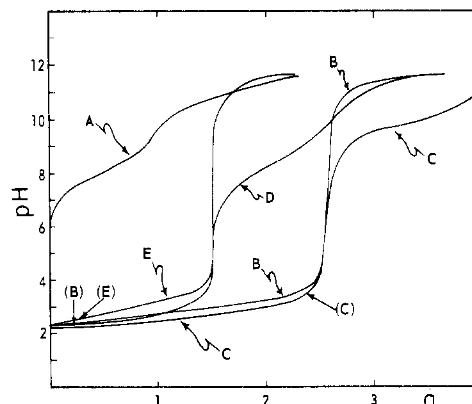



**Figure 2.** pH titration of $dl$-penicillamine and Sn(II) with potassium hydroxide: curve A, free ligand, 0.01 *M*; curve B, 0.01 *M* Sn(II) + 0.005 *M* HCl; curve C, 0.01 *M* ligand + 0.01 *M* Sn(II) + 0.005 *M* HCl; curve D, 0.01 *M* ligand + 0.005 *M* Sn(II) + 0.005 *M* HCl; curve E, 0.005 *M* Sn(II) + 0.005 *M* HCl. Ionic strength $\mu$ = 0.1 *M* (KCl); $a$ = added equivalents of potassium hydroxide. The straight arrows B, C, and E indicate onset of precipitation.

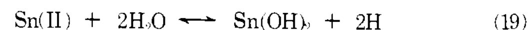
$a$ = 0 to $a$ = 0.5, hydrochloric acid is neutralized. The neutralization curve ranging from $a$ = 0.5 to $a$ = 2.5 can be explained on the basis

$$Sn(II) + 2H_2O \longrightarrow Sn(OH)_2\downarrow + 2H \quad (18)$$

although the white precipitate, starting around pH 2.3, presumably contains also species of the composition $[Sn(OH)]^+$, $[Sn_2(OH)_2]^{2+}$, and $[Sn_3(OH)_4]^{2+}$ (Tobias, 1958).

Titration of Sn(II) in the presence of an equimolar concentration of penicillamine yields curve C. Curve C differs from curve B (Figure 2) in that it proceeds from $a$ = 0 to $a$ = 2.5 at somewhat lower pH values (lowered by about 0.3–0.5 pH units) and also that a white precipitate is formed only at $a \sim 2.5$ and pH $\sim 3.5$. A further deviation occurs at $2.5 < a < 4$, where curve C exhibits a new buffer region stretching from about pH 9 to 11.

Curve C can be interpreted (after neutralization of excess HCl from $a$ = 0 to $a$ = 0.5) as representing complex formation either according to reaction 15 (from $a$ = 0.5 to $a$ = 2.5, Figure 2) or, in a first approximation, according to



$$Sn(II) + 2H_2O \longrightarrow Sn(OH)_2 + 2H \quad (19)$$
$$Sn(OH)_2 + H_2L \longrightarrow SnL + 2H_2O$$

which obviously yields the same overall hydrogen ion balance. In an attempt to see whether or not tin hydroxo complexes of the form $[Sn(OH)]^+$, $[Sn_2(OH)_2]^{2+}$, $[Sn_3(OH)_4]^{2+}$, or $Sn(OH)_2$ are in existence in the presence of penicillamine (or cysteine) in the range $0.5 < a < 2.5$, the $\beta_1^{Sn}$ values of both amino acids were evaluated using at one time eq 9–11, together with the appropriate tin hydroxo complex formation constants (Tobias, 1958), and at another time by employing the simplified eq 16 and 17. The excellent agreement of the various $\beta_1^{Sn}$ constants with one another (*cf.* Table II) indicates to us that both of the expressions $\Sigma_{q=1}\Sigma_{r=1}q[M_q(OH)_r]$ and $\Sigma_{q=1}\Sigma_{r=1}r[M_q(OH)_r]$ can be neglected in the evaluation and that chelation proceeds also in the case of Sn(II) more or less according to reaction 15. This interpretation is further supported by the results of our polarographic studies at pH 2.5, which also show the absence of tin hydroxo complexes in the presence of the sulfur-containing amino acids (Gruenwedel and Patnaik, 1972a). We did not investigate the part of curve C pertaining to the buffer re-

gion $2.5 < a < 4$. Presumably it involves reactions of the type $SnL + H_2O \rightarrow Sn(OH)L + H$.

Of considerable interest is curve D (Figure 2), the neutralization curve of a mixture composed of Sn(II) and penicillamine in the molar ratio 1:2. After neutralization of excess HCl ($a = 0$ to $a = 0.5$), there exists a buffer region ($a = 0.5$ to $a = 1.5$) indicating chelate formation only according to $Sn(II) + H_2L \rightarrow SnL + 2H$, despite the twofold excess of penicillamine (*cf.*, for instance, Figure 1, curve B, and Figure 2, curve D). Also, in contrast to curve C, no precipitation is observed over the entire pH range. Evaluation of curve D in the range $a = 0.5$ to $a = 1.5$ yields log $\beta_1^{Sn} = 16.29$, which is in close agreement with the other $\beta_1^{Sn}$ values listed in Table II. The apparently exclusive formation of 1:1 Sn(II)-cysteinato (and penicillaminato) chelates under the experimental conditions employed in this work is also indicated by the results of our polarographic studies at pH 2.5 and in 0.1 *M* KCl, where the existence of 1:2 chelates could only be detected at ligand concentrations that were at least in a 50-fold excess over the Sn(II) concentration (Gruenwedel and Patnaik, 1972a). If we accept this interpretation, then the buffer region stretching from $a = 1.5$ to $a = 3$ (curve D) should be composed of the overlap of the reactions $H_2L \rightarrow HL + H \rightarrow L + H$ (excess amino acid) and $SnL + H_2O \rightarrow Sn(OH)L + H$ (*cf.*, curve C), which yields a total of 3 H equivalents. Our titration curve indicates consistently the liberation of 2.6–2.7 H equivalents ($a = 1.5$ to $a = 2.8$). We are at the moment unable to assess the significance of this deviation. It is known that reactions of the type $ML + H_2O \rightarrow M(OH)L + H$ are often kinetically inhibited so that the measured endpoint might not be identical with the equilibrium endpoint. In addition, metal-cysteinato and penicillaminato chelates have been observed to undergo disproportionation reactions according to $2ML + 2H_2O \rightarrow ML_2 + M(OH)_2\downarrow + 2H$ (Doornbos, 1968; Doornbos and Faber, 1964), which might also result in kinetic inhibitions. We did not investigate this buffer region of curve D any further, since we feel that methods other than pH titration techniques (*e.g.*, Raman spectrophotometry, nuclear magnetic resonance, etc.) are by far better suited to study the reaction in this pH range.

The titration curves comprising Sn(II) and cysteine are essentially the same as the ones comprising tin and penicillamine. We therefore did not include them in this work.

**Conclusions.** Two interesting observations, it seems to us, have thus far emerged from this investigation: (a) the fact that cysteine as well as penicillamine binds Sn(II) ions much more strongly than Fe(II) ions, and (b) that the binding of Sn(II) occurs already at low pH values. It is evident that in the case of iron, the $\alpha$-carboxyl group of the amino acids has to be completely ionized before metal binding occurs. Thus, pH titrations are here unsuitable to demonstrate whether or not this group participates in chelation. In the case of Sn(II), however, the $\alpha$-carboxyl group is partially still protonated when chelation proceeds and here it appears as if the group is also engaged in metal binding. Preliminary infrared data (Gruenwedel and Patnaik, 1972b) indicate that with Sn(II) both cysteine and penicillamine behave as terdentate ligands (through a carboxyl oxygen, the nitrogen, and the sulfur atom). It has been suggested that tin(II) is four-coordinated in certain chelates and $p^3d$ hybrid orbitals are involved in the bonding (Smith, 1961). This would explain the predominance of 1:1 chelates with cysteine and penicillamine and also additional reactions of the type $SnL + H_2O \rightarrow Sn(OH)L + H$ at pH values above 9; three of the four sites around the central metal ion are then occupied by an amino acid and the fourth site is occupied by the OH group.

Penicillamine forms complexes both with iron(II) and tin(II) ions, which are somewhat more stable than the ones formed by cysteine. By contrast, protons appear to be bound by the two ligands only with equal strength.

Although there is evidence to suggest that cysteine in particular readily forms polynuclear complexes besides 1:1 and 1:2 chelates [*e.g.*, in the case of Ni(II) (Perrin and Sayce, 1968), Zn(II) (Perrin and Sayce, 1968; Shindo and Brown, 1965), Cd(II), Hg(II), and Pb(II) (Shindo and Brown, 1965)], no such evidence seems to exist in the case of Sn(II). Also with Fe(II), no polynuclear complexes have been reported thus far (Doornbos, 1968; Doornbos and Faber, 1964; Tanaka *et al.*, 1955).

Of practical importance to the food technologist is of course the finding that sulfur-containing amino acids form very stable chelates with tin(II) already at low pH values, whereas iron (II) becomes chelated only at pH > 6. To our knowledge, only Hg(II) and, to a lesser extent, also Pb(II) appear to react with penicillamine and cysteine already at low pH values (Doornbos and Faber, 1964). The pH frequently found in many low-acid foods such as seafood products, vegetables, and meat is around 6. At this pH, an iron–cysteinate solution is completely dissociated into $Fe^{2+}$ and cysteine. A tin-cysteinate solution, on the other hand, is dissociated into its components at the same pH, only by a factor of $10^{-8.3}$. The limiting concentration ratio $[Sn^{2+}]/[Fe^{2+}] \leq 5 \times 10^{-11}$ mentioned above, at which the electromotive force of a tin electrode is equal to the electromotive force of an iron electrode, is a value based solely on equilibrium conditions and therefore neglects all polarization effects, which certainly will have to be taken into account when evaluating the true behavior of the tin-iron couple in food cans. Thus, we do not deem it particularly fruitful to analyze the stability data in any further detail as far as the reversal of polarity is concerned. However, there can be no doubt that our findings are in agreement with the observation by Pigott and Dollar (1963) that tin is anodic to iron also in low-acid foods and, further, that they substantiate rather convincingly the concept of a reversal in polarity based on chelate formation (Hoar, 1934).

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# Extraction of Soybean Meal Proteins with Salt Solutions at pH 4.5

Robert L. Anderson, Walter J. Wolf,* and Donald Glover[1]

Extractable Kjeldahl nitrogen increased with increases in concentration of sodium or calcium chloride until a maximum of 65% of the nitrogen in the flakes was extracted. This maximum occurred with 0.3 *N* calcium chloride or 0.7 *N* sodium chloride. Without added salts, the pH 4.5 extract contained only 2S and 7S ultracentrifuge components. Up to 0.3 *N* sodium chloride 2S protein increased, whereas the 7S component did not reach a maximum until 0.7–0.8 *N* salt. The 11S component began to dissolve at 0.3 *N* salt and was completely solubilized at 0.8 *N*. The 15S component did not dissolve until concentrations of salt were greater than 0.4 *N* and increased in extractability up to 0.8 *N* sodium chloride. Calcium chloride extracts contained increasing amounts of 2S and 7S fractions up to 0.2 and 0.3 *N*, respectively. The 11S component began to dissolve at 0.1 *N* and increased in solubility up to 0.4 *N* calcium chloride. The 15S material did not dissolve significantly below 0.2 *N* and increased in extractability up to 0.4 *N* calcium chloride.

Distilled water extracts of defatted soybean meal have a pH of 6.5–6.7 and contain about 80–90% of the meal proteins (Smith *et al.*, 1938). When extractions are carried out with dilute salt solutions near this pH, extractability of the proteins depends upon salt concentration. As the concentration increases less protein is dissolved, until a minimum of about 46% of the total Kjeldahl nitrogen is extracted with 0.1 *N* sodium chloride and only about 22% is extracted with 0.018 *N* calcium chloride. After the minimum in solubility is reached, more protein dissolves as the salt concentration of the extraction solvent is further increased. In this respect, soybean proteins are unique as compared to the proteins of flax, rye, wheat, and barley (Smith *et al.*, 1938). If the pH is lowered to the isoelectric region of 4.6 during water extraction of soybean proteins, only 9% of the total Kjeldahl nitrogen dissolves (Smith and Circle, 1938). Adding salts during extraction at this pH increases dispersion of the protein. This increase contrasts with the minimum in extractability found near neutral pH.

Ultracentrifugal analysis of water-extractable soybean proteins reveals four components with sedimentation coefficients of approximately 2, 7, 11, and 15S (Svedberg units). Changes occur in the distribution of these components as a function of sodium and calcium chloride concentrations used for extraction near neutral pH (Wolf and Briggs, 1956). Preferential extraction of some proteins occurs as salt concentrations are varied. We examined extractability of soybean meal proteins at pH 4.5 as a function of sodium and calcium chloride concentrations and determined the ultracentrifugal composition of the various extracts.

Northern Regional Research Laboratory, U. S. Department of Agriculture, Peoria, Illinois 61604.
[1]Department of Chemistry, Bradley University, Peoria, Illinois 61606.

## EXPERIMENTAL SECTION

**Protein Extraction Procedure.** Harosoy soybeans (1964 crop) were cracked, dehulled, and flaked. The flakes were defatted by four batchwise extractions with pentane–hexane (boiling range 33–57°) and then air dried. Nitrogen content of the defatted flakes was 9.1%, corrected for 10.6% moisture. Protein extracts were prepared with a ratio of 20 ml of extractant/g of defatted flakes, except in preliminary experiments in which a 40:1 extraction ratio was used. After addition of the extractant to the flakes, the pH was quickly adjusted with 1 *N* HCl to 4.5 and maintained while stirring for 45 min. The slurry was then centrifuged for 15 min at 19,000 × *g*. The resultant supernatant was filtered through Whatman No. 1 filter paper to remove a small amount of floating material. Nitrogen analyses were performed on the filtered extracts.

**Ultracentrifugation.** A 4-ml portion of extract was equilibrated by dialysis against pH 7.6, 0.5 ionic strength, phosphate buffer containing 0.01 *M* 2-mercaptoethanol (Wolf and Briggs, 1959). Preliminary studies indicated no changes were brought about in the samples by dialysis against the phosphate buffer. Calcium chloride extracts were first dialyzed against 0.4 *N* sodium chloride to remove the calcium ions before dialysis against phosphate buffer. After dialysis and centrifugation for 15 min at 19,000 × *g*, the extracts were diluted to 5 ml with buffer.