Protective Effect of Casein on Cleavage of Thiamine by Sulfite

Joseph Leichter and M. A. Joslyn

Previously reported data indicated that casein exerts a protective effect on the cleavage of thiamine by sulfite. Binding of added sulfite, which occurs, does not account for all the protective effect. Catalysis by casein of the oxidation of sulfite by oxygen was observed to occur. Splitting of casein disulfide bonds by sulfite was believed to be a factor but the addition of cystine, expected to have a sparing effect on this, actually did not protect thiamine against cleavage by sulfite. In view of the highly protective effect of soluble starch on thiamine cleavage it is believed that some macromolecular property, *e.g.* interfacial surface effect, was involved in the protective effect observed. Neither thiamine nor sulfite were found to combine with casein in appreciable concentrations under the conditions used. The thiamine cleavage by sulfited casein is water dependent.

Sulfite has been found to be reasonably effective in preventing the browning reaction (Mapson and Wager, 1961). It is also known to split thiamine into biologically inactive components (Williams *et al.*, 1935). Because of these properties, sulfite has been used to provide stable products of light color, and in preparing dietary constituents free of thiamine for bioassay purposes (Mapson and Wager, 1961; The Mogul Corp., 1968). The use of sulfite for the latter purpose sometimes leads to undesirable results. Miller *et al.* (1955) showed that the purified diet which caused symptoms similar to those of vitamin E deficiency in chicks, previously reported by Carlson *et al.* (1949), contained variable amounts of sodium bisulfite present in the commercially prepared "alpha protein" (isolated soybean protein) as a consequence of manufacturing procedure. They showed that the sulfite had an adverse effect on the stability of vitamin E and suggested that the tremors and paralysis in chicks reported by Carlson *et al.* (1949) may have been caused at least partially by thiamine deficiency due to the presence of sulfite in the alpha protein.

The authors showed in a previous paper (Joslyn and Leichter, 1968) that residual sulfite present in some commercially available vitamin-free caseins was responsible for the cleavage of thiamine during storage in aqueous suspensions. The rate of thiamine cleavage by sulfited casein in aqueous suspension was considerably slower than at an equivalent concentration of sulfite solution but in the absence of casein, indicating an unknown protective effect of casein. The present paper deals with the nature of this protective effect of casein.

MATERIALS AND METHODS

The thiamine hydrochloride used was a USP preparation, the sodium metabisulfite a reagent grade chemical, and the soluble starch, reagent grade, (improved Lintner Method) from Pfanstiehl Chemical Co., Waukegan, Ill. Vitamin-free casein was obtained from General Biochemicals, Inc., Chagrin Falls, Ohio, and from Calbiochem, Los Angeles, Calif.

Total sulfur dioxide determinations were made by both the Monier-Williams method of the AOAC (1965), and the modified colorimetric procedure of Nury *et al.* (1959). For the determination of free sulfur dioxide in sulfited casein, the method of Lloyd and Cowle (1963) was followed, using the colorimetric procedure. Thiamine was determined by the thiochrome method of the Association of Vitamin Chemists (1966) omitting the enzyme digestion and column purification steps.

To determine the rate and extent of cleavage of thiamine by the residual sulfite present in casein, 20% aqueous suspensions of casein were prepared containing 200 μg. of thiamine per 100 grams of suspension at a pH of 5.5. These suspensions were held in a water bath at 25° C. To prevent microbial growth, 100 p.p.m. merthiolate (Eli Lilly and Co., Indianapolis, Ind.) were added to the casein suspensions. Merthiolate did not interfere with the thiochrome method of thiamine determination.

To determine the adsorption of thiamine by casein, sulfite-free casein was used (Calbiochem). Two series of experiments were carried out in which either the concentration of sulfite-free casein was constant and the concentration of thiamine varied or the thiamine held constant and the casein concentration varied. The prepared suspensions were shaken for 10 minutes in screw cap centrifuge tubes and then centrifuged in a Beckman, Model L-2 preparative ultracentrifuge for three hours at 30,000 r.p.m. and 25° C. The thiamine content was subsequently determined on both the sediment and the supernatant, and the results expressed in terms of micrograms thiamine per gram of casein. The same technique was used to determine the combination of sodium metabisulfite with casein.

To test the effect of moisture on rate of thiamine cleavage by sulfited casein, a well-mixed preparation containing 20 grams of sulfited casein (12 mg. of SO_2 per 100 grams of suspension) and 300 μg. of thiamine per 100 grams of casein suspension was lyophilized (VirTis Manifold Freeze-Dryer). The lyophilized material was powdered and thoroughly mixed. Thin layers of these lyophilized preparations in petri dishes (50 grams) or weighing bottles (1 to 2 grams) were placed above sulfuric acid solutions (350 ml.) of the desired concentration in closed desiccators, previously equilibrated. The desiccators were held in a constant temperature water-bath at 25° C. Aliquots for residual thiamine and sulfur dioxide determination were removed periodically. The weighing bottles were weighed daily to determine moisture gain by the lyophilized preparations and replaced above the sulfuric acid. When the test was concluded (after 3 weeks), the sulfuric acid in the desiccators was, after appropriate dilutions, titrated with standard alkali. From the concentrations of the acid samples, the relative humidities were obtained in accordance with the International Critical Tables (NRC, 1928).

To determine whether oxidation of sulfite to sulfate occurred during thiamine cleavage by sulfited casein suspensions, the increase in sulfate content was determined. Aliquots of sodium metabisulfite equivalent to 100 mg. of sulfur dioxide

Department of Nutritional Sciences, University of California, Berkeley, Calif. 94720

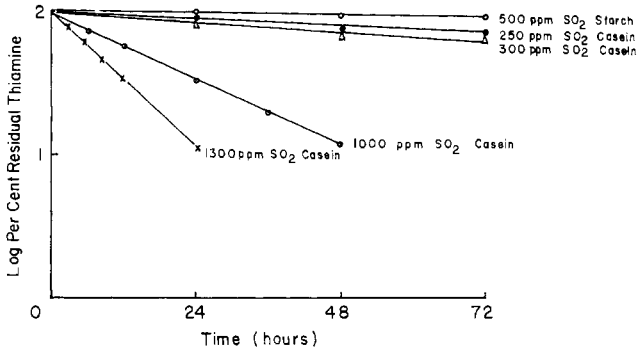

Figure 1. Rate of thiamine cleavage by various concentrations of sulfite in presence of casein and soluble starch

Thiamine level 200 μg. per 100 grams suspension, casein and starch levels 20 wt. %, pH 5.5 at 25° C.

were added to solutions of thiamine chloride (200 μg. per 100 grams) in presence and absence of casein (20 grams of sulfite-free casein per 100 grams of suspension). The distilled water used was freshly boiled and cooled under a stream of carbon dioxide and the flasks were swept out with carbon dioxide before adding the solution. The mixture was adjusted to pH 5.5 and then shaken in a glass-stoppered Erlenmeyer flask for 10 minutes and stored at 25° C. for 6 hours. The sulfate was extracted from the mixture by adding 100 ml. of acid 25 per cent potassium chloride and filtering through Whatman No. 1 filter paper. Then 5 ml. of 1*N* HCl were added to the total filtrate and boiled for 10 minutes followed by the addition of 10 ml of 10% barium chloride solution and boiling for 15 minutes. After standing overnight, the mixture was filtered on ashless paper, washed free from chlorides, ignited at 600° C., cooled in a desiccator, and weighed.

To test the effect of disulfide bonds (—S—S—) on the cleavage of thiamine by sulfite, 6 and 60 μ*M* of cystine were added to a solution of thiamine chloride (6 μ*M* per 100 ml) in presence of sulfited casein (20 grams of 1000 p.p.m. SO_2 casein per 100 grams suspension). A similar test was conducted in the absence of casein with 1 m*M* SO_2. The pH of the mixtures was adjusted to 5.5 and the storage temperature was 25° C. The thiamine determinations were done after 0, 6, 12, and 24 hours. The procedure used was that as described above.

All the assays were conducted in duplicate and the average values are reported. Variations between duplicate samples rarely exceeded 3%. The reaction conditions for each test are shown in the respective figures and tables.

The sulfite present is expressed as sulfur dioxide. The specific reaction rate constants were calculated for the linear portion of the data plotted as a monomolecular reaction.

RESULTS AND DISCUSSION

To four 20% casein suspensions and to one 20% soluble starch suspension containing different amounts of sulfite at pH 5.5 and 25° C. 200 μg. of thiamine were added per 100 grams of solution. When logarithms of the percentages of residual thiamine were plotted against time, the resulting graphs were linear as shown in Figure 1. The calculated specific reaction rate constants for thiamine cleavage by sulfite, and the total and free sulfur dioxide content in the four lots of vitamin free casein and soluble starch are shown in Table I. Though the total SO_2 levels in samples C and D are 4 to 5 times higher than in samples A and B, the combined SO_2 is only twice as high. It appears that the ability of casein to combine with sulfite is saturated in samples C and D, whereas in samples A and B it did not reach the maximum possible limit. In the casein samples, the increase in the specific reaction rate constants is not proportional to the amount of sulfite present. However the specific reaction rate constants of thiamine destruction by sulfite under identical conditions, but in the absence of casein, also shown in Table I, are not only significantly greater than in the presence of casein but they also increase proportionally with the amount of sulfite. Since the soluble starch (Pfanstiehl Chemical Co.) suspension also shows a protective effect on thiamine destruction by free sulfite (Table I), it appears that the protective effect of casein on thiamine is partially due to its macromolecular property, *e.g.*, intrafacial surface effect as well as to its binding capacity for sulfite.

The effect of other materials on the rate of thiamine cleavage by sulfite was also tested. Beside the soluble starch these included cellulose powder, standard grade (Whatman), gelatin (DIFCO Laboratories), sucrose, purified sand, corn starch, potato starch, wheat starch powder, and rice starch. Except for the protective effect of soluble starch and partially corn starch on the rate of thiamine cleavage by sulfite, all the other materials showed a negligible or no effect.

The data on thiamine destruction in presence of various concentrations of casein (sample C) are shown in Figure 2. The destruction of thiamine in all the casein suspensions was first order with respect to thiamine. The specific reaction rate constants calculated from the graphs (Figure 2) increase with the amount of sulfite present in the casein suspensions. The specific rate constant increased from 255 × 10^{-4} $hr.^{-1}$

Table I. Total and Free Residual Sulfite Present in Different Samples of Casein and Specific Reaction Rate Constants of Thiamine[a] Cleavage by Various Concentrations of Sulfite in Presence and Absence of Casein and/or Starch

Product	Total SO_2, p.p.m.	Free SO_2, %	Rate Constant,[b] *k*	Rate Constant,[c] *k*	Rate Constant,[d] *k*
Vitamin-free casein					
A	250	46	40 × $10^{-4}hr.^{-1}$	380 × $10^{-4}hr.^{-1}$	180 × $10^{-4}hr.^{-1}$
B	300	48	54 × $10^{-4}hr.^{-1}$	450 × $10^{-4}hr.^{-1}$	230 × $10^{-4}hr.^{-1}$
C	1000	72	440 × $10^{-4}hr.^{-1}$	1450 × $10^{-4}hr.^{-1}$	1050 × $10^{-4}hr.^{-1}$
D	1300	80	780 × $10^{-4}hr.^{-1}$	1900 × $10^{-4}hr.^{-1}$	1500 × $10^{-4}hr.^{-1}$
Soluble starch	500	100	29 × $10^{-4}hr.^{-1}$	546 × $10^{-4}hr.^{-1}$	546 × $10^{-4}hr.^{-1}$
SO_2 free casein	0	—	0	—	—

[a] Thiamine concentration 200 μg. per 100 grams suspension at pH 5.5 and 25° C.
[b] Casein and starch were present at levels of 20 wt. %, thiamine concentration was 200 μg. per 100 grams suspension, pH 5.5, at 25° C.
[c] The conditions were identical as in [b] but in the absence of casein or starch.
[d] As in [c] but the sulfite concentrations correspond to the free SO_2 present in the casein samples.

at 5% casein suspension to 1120 × 10^{-4} hr.$^{-1}$ at 25% casein suspension.

The sulfite content present in the sulfited caseins was found to be surprisingly stable when the samples were stored at room temperature for as long as a year. However, an appreciable change in sulfite content occurred in 20% casein suspensions kept at 25° C., particularly when caseins with higher levels of sulfite (samples C and D) were used. On the average, a 20 and 40% decrease in SO_2 occurred in 20% casein suspensions with samples C and D after one and three days, respectively.

The oxidation of sulfite to sulfate during cleavage of thiamine by sulfite after a 6-hour period is shown in Table II. More sulfate accumulated during cleavage of thiamine in presence than in absence of casein. In cleavage of thiamine by sulfite, pyrimidine sulfonic acid is produced and no free sulfate accumulates. Therefore the protective effect of casein on the cleavage of thiamine by sulfite could be due, in part, to increased oxidation of sulfite to sulfate.

That oxidation of sulfite in presence of casein was a factor in decreased cleavage of thiamine also was shown from data comparing cleavage rate in presence of added sulfite to those obtained with sulfited casein. The specific rate constant of thiamine cleavage by sulfite carried out in a 20% casein suspension prepared from a casein containing 400 p.p.m. SO_2 was 100 × 10^{-4} hr.$^{-1}$. When the equivalent amount of SO_2 was added to a sulfite-free casein the specific rate constant was 55 × 10^{-4} hr.$^{-1}$. The initial thiamine concentration present was 200 μg. per 100 grams at pH 5.0 at 25° C. This decrease in rate of thiamine cleavage was accompanied by a marked decrease in SO_2 content. The total SO_2 content in the run made with sulfited casein decreased from 60 to 57.5, 57.5, and 47.5 p.p.m., respectively after 1, 2, and 3 days. In the run with added sulfite, the SO_2 content decreased from 42.5 to 20, 15, and 12.5 p.p.m., respectively, after 1, 2 and 3 days. This decrease in available SO_2 content could account for the difference in rate observed since in SO_2 solutions the calculated specific rate constants would be 320 × 10^{-4} hr.$^{-1}$ at 42.5 p.p.m. and 160 × 10^{-4} hr.$^{-1}$ at 20 p.p.m.

The protective effect of casein on the cleavage of thiamine by sulfite could be due, in part, to competitive reaction of the disulfide linkage in casein with sulfite (Cecil, 1963). Wallace and Aiyar (1969) estimated the sulfhydryl and disulfide groups in casein. They reported the presence of both cysteine and cystine in whole casein in the proportions of 1:1 by weight and in the molecular ratio of 1:2. On this basis, our reaction mixture usually contained 0.3 equivalents of cystine and 0.0006 equivalents of thiamine per 0.1 equivalent of SO_2. However, when 1 and 10 equivalents of cystine were added to thiamine solution in absence, and in presence of casein, no decrease in rate of thiamine cleavage was observed after a 24-hour period at pH 5.5 and 25° C. While the optimum pH for splitting of disulfide bonds by sulfite is given as 7.0 or higher (Cecil and McPhee, 1955), Wallace and Aiyar (1969) found that conversion of cystine into cysteine at pH 6.5–7.0 by excess of sodium sulfite is complete in 1 hour at room temperature. Cecil and McPhee (1955) indicate that the reaction below pH 9 is complicated by ionization of amino groups and of bisulfite ions. Apparently under our conditions reaction of sulfite with cystine is not competitive with that of thiamine.

That sulfite does reduce the disulfide groups in casein was shown by comparing the S—S and S—H content of unsulfited casein (Calbiochem) before and after treatment with

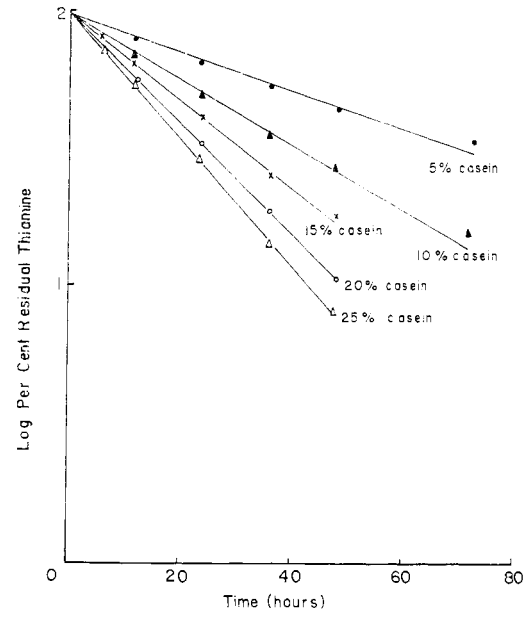

Figure 2. Rate of thiamine cleavage by sulfite in presence of various concentrations of sulfited casein

Thiamine level 200 μg. per 100 grams suspension, Sample C casein was used (1000 p.p.m. SO_2), pH 5.5 at 25° C.

Table II. Oxidation of Added Sulfite to Sulfate in Presence and Absence of Casein[a]

Casein[b] (grams)	Water (ml.)	Sulfite Added (as SO_2 mg.)	Sulfite Oxidized (as SO_2 mg.)
0	100	100	6.5
20	80	100	12.8
20	80	0	4.0

[a] Contact time 6 hours, at pH 5.5 and 25° C.
[b] Casein, sulfite-free, from Calbiochem, Los Angeles.

5000 p.p.m. SO_2 solution for 3 days at room temperature with values observed for sulfited casein (General Biochem, 1300 p.p.m.). The cysteine and cystine content expressed in milligrams per 100 grams of these samples as determined by the method of Wallace and Aiyar (1969) was as follows:

Casein Sample	Cysteine mg/100 g	Cystine mg/100 g
Unsulfited	133	260
Unsulfited after treatment with sodium metabisulfite in 20% suspension	327	95
Sulfited	242	151

Adsorption of the added thiamine or bisulfite by casein was investigated as a possible factor to explain the protective effect of casein. Inagaki and Fukuba (1959) studied the adsorption of thiamine by frozen bean-curd and other proteins. When frozen bean-curd, thawed and pressed to dehydrate, was dipped into solutions containing various amounts of thiamine, about 80 to 90% of the thiamine was adsorbed by the bean-curd. The recovery of the thiamine adsorbed was very low when determined directly. However the thiamine recovery was complete when the bean-curd was treated with proteinase. From this Inagaki and Fukuba (1959) assumed that the thiamine adsorbed by bean-curd is combined with

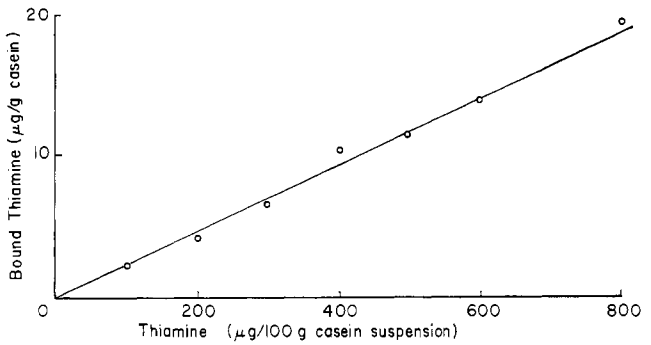

Figure 3. Binding of thiamine by 10% sulfite-free casein suspensions at thiamine levels from 0 to 800 μg. per 100 grams suspension

pH 5.5 at 25° C.

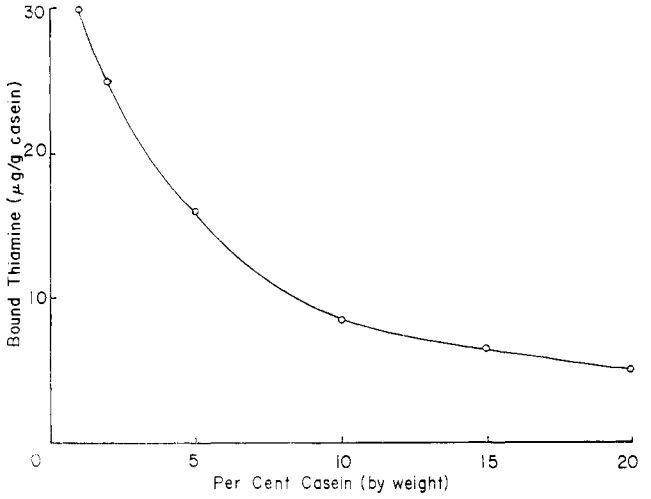

Figure 4. Binding of thiamine by casein at casein concentrations from 0 to 20% by weight and thiamine at a level of 600 μg. per 100 grams suspension

pH 5.5 at 25° C.

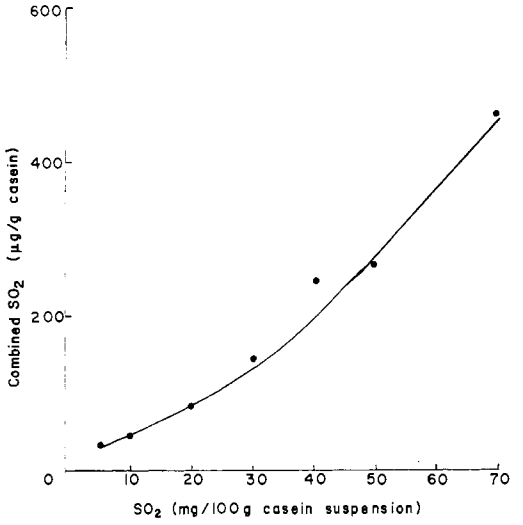

Figure 5. Binding of sulfite by 10% sulfite-free casein suspensions at SO_2 concentrations from 5 to 70 mg. per 100 grams suspension at pH 5.5 and 25° C.

the protein and could not be extracted completely with 1*N* HCl.

In view of the above, we decided to study the adsorption of thiamine by casein at varying concentrations of both thiamine and casein. The data on the adsorption of thiamine by sulfite-free casein are presented in Figures 3 and 4. The

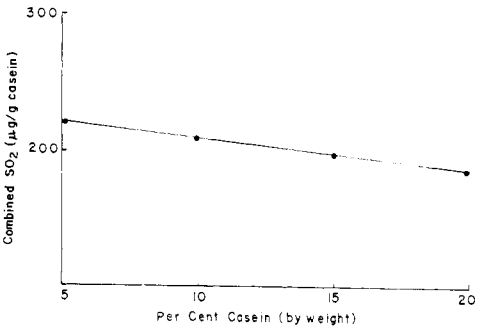

Figure 6. Binding of sulfite 500 p.p.m. as SO_2 by sulfite-free casein at casein concentrations from 5 to 20% by weight at pH 5.5 and 25° C.

Table III. Data on Adsorption or Combination of Sulfite with Vitamin-Free Casein

Casein, %	SO_2, μg. per 40 Grams	Supernatant Portion, μg. SO_2 Found	Sediment, μg. SO_2 Found	Supernatant Portion Plus Sediment, μg. SO_2	Recovery, %
0	20000	19000	0	19000	95
5	20000	16600	1120	17720	88
10	20000	16500	2070	18570	93
15	20000	14300	2900	17200	86
20	20000	12100	3700	15800	79
10	2000	1160	275	1435	73
10	4000	2530	385	2915	70
10	8000	6090	825	6915	86
10	12000	8700	1430	10130	84
10	16000	16575	2420	18995	118
10	20000	17400	2530	20930	104
10	28000	30450	4510	34960	124
10	36000	39150	6105	45255	125

thiamine concentration was determined only in the supernatant portion and data on thiamine recoveries were not obtained. In Figure 3, casein was kept constant (10% sulfite-free casein suspension) and thiamine concentrations varied from 0 to 800 μg. per 100 grams suspension. In Figure 4, thiamine was kept constant at a level of 400 μg. per 100 grams suspension and casein concentrations varied from 0 to 20% by weight. The data plotted in Figure 3 indicate that the thiamine combined with casein increased linearly with concentration of thiamine present. The plot, however, did not follow the usual Langmuir adsorption isotherm, but the thiamine concentrations used were too low relative to casein present to be sure. In Figure 4, the thiamine combined with casein decreased as the concentration of casein was increased. Appreciable adsorption of thiamine by casein apparently did not occur and the protective effect of casein on thiamine cleavage by sulfited casein could not be ascribed to this.

It is of interest that more thiamine was bound per gram of casein in dilute than in more concentrated suspensions. This could be due to the more ready availability of active surface at the lower concentration or to the fact that hydration was more uniform and greater at the lower levels. Hydration of casein is a factor in determining thiamine cleavage. As the data in Figure 7 show, no cleavage occurred in dry caseins and cleavage increased in rate and extent as hydration increases.

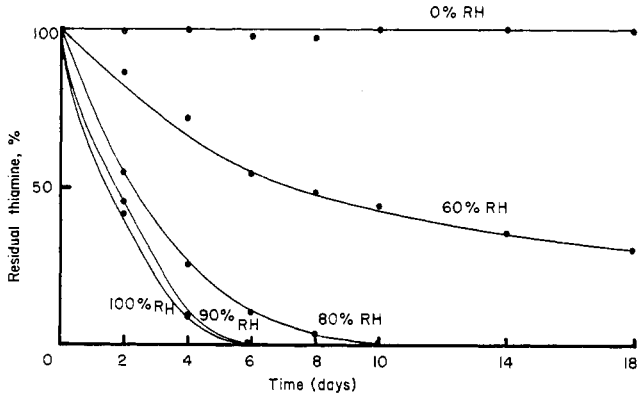

Figure 7. Rate of thiamine cleavage by sulfite in lyophilized casein held at various relative humidities

SO_2 concentration was 600 p.p.m. and thiamine 15 p.p.m. at 25° C.

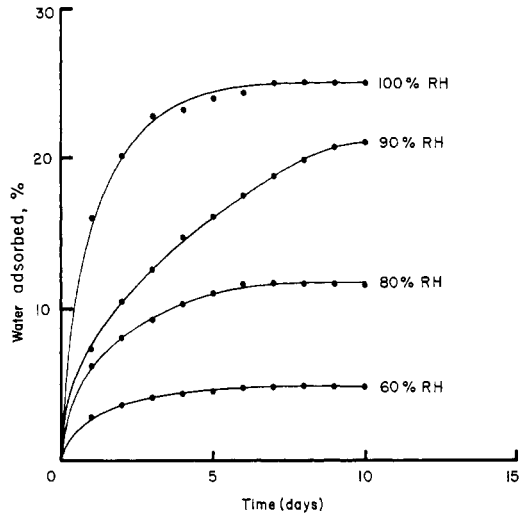

Figure 8. Rate of water adsorbed by lyophilized casein held at various relative humidities at 45° C.

The adsorption or combination of sodium metabisulfite with sulfite-free casein was investigated under similar conditions. Either the casein was kept constant (10% sulfite-free casein suspension) and sulfur dioxide concentration varied from 5 to 90 mg. per 100 grams suspension or the sulfur dioxide was held constant and the casein concentration varied from 0 to 20% by weight. The results obtained are presented in Figures 5 and 6 and Table III. As with thiamine appreciable adsorption of sulfite by casein apparently did not occur. The recoveries of SO_2 were variable and low (Table III). In casein suspensions containing low levels of SO_2 (12 mg. SO_2 per ml. or less) recovery varied from 70 to 80%. When the levels of SO_2 were 16 mg. per ml. or above, the recovery was higher than the calculated amount added. This occurred in the runs where the concentration of SO_2 was varied and that of casein was kept constant. The results presented in Figures 5 and 6 are influenced both by irregularities in the recovery of total SO_2 and by the loss of SO_2 due to oxidation.

Figure 7 shows the effect of moisture on thiamine cleavage by sulfite when lyophilized samples of sulfited casein containing 600 p.p.m. SO_2 and 15 p.p.m. thiamine were placed at different relative humidities. No destruction of thiamine occurred even after 3 months at 0% relative humidity. Complete destruction of thiamine occurred after 6, 6, and 10 days when the lyophilized samples were kept at 100, 90, and 80% relative humidities, respectively, and 70% thiamine destruction in 18 days at 60% relative humidity. It should be pointed out that the thiamine in the lyophilized samples was completely destroyed during the period of rapid increase in moisture in 100 and 90% relative humidity, and 90% of thiamine in the case of 80% relative humidity, as can be seen by the comparative water adsorption by the lyophilized samples in Figure 8.

LITERATURE CITED

Association of Official Agricultural Chemists, "Official Methods of Analysis," 10th ed., p. 466, 27.077, 1965.

Association of Vitamin Chemists, "Methods of Vitamin Assay," p. 127, Interscience Publishers, Inc., New York, N. Y., 1966.

Carlson, C. W., Miller, R. F., Peeler, H. T., Norris, L. C., Heuser, G. F., *Poultry Sci.* **28,** 750 (1949).

Cecil, R., in "The Proteins," (H. Neurath, Ed.), 2nd ed., Vol. 1, p. 386, Academic Press Inc., New York and London, 1963.

Cecil, R., McPhee, J. R., *Biochem. J.* **60,** 496 (1955).

Inagaki, C., Fukuba, H., *Bitamin.* **17,** 17 (1959).

Joslyn, M. A., Leichter, J., *J. Nutr.* **96,** 89 (1968).

Lloyd, W. J. W., Cowle, B. C., *Analyst* **88,** 394 (1963).

Mapson, L. W., Wager, H. G., *J. Sci. Food Agr.* **12,** 43 (1961).

Miller, R. F., Small, G., Norris, L. C., *J. Nutr.* **55,** 81 (1955).

Mogul Corporation, Life Science Divisions, Chagrin Falls, Ohio, personal communication, July 1968.

National Research Council, "International Critical Tables," **3,** 303 (1928).

Nury, F. S., Taylor, D. H., Brekke, J. E., J. AGR. FOOD CHEM. **7,** 351 (1959).

Wallace, G. M., Aiyar, K. R., *J. Dairy Res.* **36,** 115 (1969).

Williams, R. R., Waterman, R. E., Keresztesy, J. C., Buchman, E. R., *J. Amer. Chem. Soc.* **57,** 536 (1935).

Received for review December 13, 1968. Accepted July 14, 1969. Joseph Leichter is a Graduate Trainee in Nutrition on U. S. Public Health Service Grant No. 5T1-GM1188-05.