[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

Isolation of a Copper Bearing Protein from Cow's Milk

BY WILLIAM L. DILLS AND J. M. NELSON

The presence of traces of copper in plant and animal tissues is attracting more and more attention. Not only is it being recognized as essential for the formation of hemin iron in animals[1] but it also has been found to play a role in the form of copper proteins as oxidases in plants.[2] Recently Saha and Guha[3] report the presence in animal tissues of a non-hemin iron associated with copper in the form of a copper–iron nucleoprotein complex.

Cow's milk contains from 0.09 to 0.17 mg. of inherent copper per liter.[4] Since the copper in milk appears not to be combined with the fat, it has been suggested that it probably occurs combined with the protein.[5] In the present study, a protein has been isolated from milk containing around 15% nitrogen and 0.19% copper. Dialysis experiments showed the copper in this protein to be non-ionic in form. Due to lack of sufficient material it was not possible to establish definitely that the protein isolated was absolutely pure, although comparing its copper content with those of other copper bearing proteins listed below suggests that it might have been mainly a single chemical substance. No attempt was made in the present study to see whether the milk copper protein was related in any way to the haemocuprein in blood.

Copper protein	Source	Copper, %
Laccase[6]	Juice of the lac-tree	0.15
Ascorbic acid oxidase[7]	Crook-neck squash	.15
Tyrosinase[8]	Lactarius piperatus	.23
Tyrosinase[2]	Common mushroom	.30
Tyrosinase[10]	Potato	.20
Haemocyanins[11]	Certain invertebrates	0.19–0.26
Haemocuprein[9]	Blood	0.34

No reactions were found in which the milk copper protein showed enzymatic activity. The au-

(1) C. A. Elvehjem, Physiol. Rev., 15, 471 (1935).
(2) D. Keilin and T. Mann, Proc. Roy. Soc. (London), 125B, 187 (1938); F. Kubowitz, Biochem. Z., 299, 32 (1938).
(3) Saha and Guha, Nature, 148, 595 (1941).
(4) Elvehjem, Steenbock and Hart, J. Biol. Chem., 83, 27 (1929); Sylvester and Lampitt, Anal., 60, 376 (1935).
(5) R. J. McIlray, New Zealand J. Sci. Tech., 17, 710 (1936).
(6) Keilin and Mann, Nature, 143, 23 (1939).
(7) Lovett-Janison and Nelson, THIS JOURNAL, 62, 1409 (1940).
(8) Dalton and Nelson, ibid., 61, 2946 (1939).
(9) Keilin and Mann, Proc. Roy. Soc., (London) 126B, 303 (1938).
(10) Kubowitz, Biochem. Z., 299, 32 (1938).
(11) Redfield, Biol. Rev., 9, 175 (1934).

thors are inclined to the opinion that this non-enzymatic activity cannot be attributed to denaturation during its isolation, although no definite proof for this conclusion can be offered. Other enzymes present in the milk, such as peroxidase, continued to accompany, in the active form, the milk copper-protein during several steps in the isolation process, without being inactivated. This conclusion is also favored by the fact that the process of isolating the milk protein was quite similar to those employed in the isolation of other copper proteins in their native condition. The isolation of tyrosinase from mushrooms and from potatoes, and ascorbic acid oxidase from squash, may be mentioned as examples.

It has been reported that milk shows a loss of antiscorbutic activity on standing.[12] Since ascorbic acid oxidase is a copper protein,[7] it was deemed worth while to test the milk copper protein for ascorbic acid oxidase activity. The result, however, proved to be negative. Likewise the milk copper protein showed no polyphenolase activity.

Experimental

Raw (unpasteurized) skimmed milk was used for the isolation of the copper bearing protein. Twenty-four to thirty-six hours had elapsed from the time the milk had been taken from the cow up to the time it was used. Forty liters of this milk contained before treatment about 1200 g. of solids, and this quantity of solid matter contained 4 mg. of copper.

The 40 liters of milk was made 0.3 saturated with ammonium sulfate by adding the salt gradually with stirring. The resulting mixture was allowed to stand overnight in a cool place. The clear supernatant liquid, formed on standing, was siphoned off and the remaining wet solids suspended in muslin bags and allowed to drain overnight. The liquid thus obtained, together with that obtained by siphoning, amounted to 33 liters (solution A), and contained 200 g. of solids (dry weight basis) exclusive of salts. The pH of the liquid was 6.6 and the 200 g. of solids contained 2 mg. of copper.

Solution A was made 0.7 saturated with ammonium sulfate, permitted to stand overnight, filtered and the filtrate discarded. The precipitate was dissolved in 4 liters of 0.1 M Na$_2$HPO$_4$, pH 8, yielding a clear reddish-yellow solution B. Solution B was next subjected to successive fractional precipitations with 0.2, 0.3, 0.4, 0.5 and 0.7 saturation with ammonium sulfate, dissolving the precipitates, formed in each precipitation, in 0.1 M Na$_2$HPO$_4$.

(12) Tauber, Proc. Soc. Exptl. Biol. Med., 35, 422 (1931).

In this way solutions C, D, E, F, and G, described in Table I, were obtained.

TABLE I

Soln.	Satn. amt. of $(NH_4)_2SO_4$ used	Vol., cc.	Color	Solids exclusive of salts, g.	Mg Cu	% Cu
C	0.2	295	Tan, turbid	17.0	0.12	0.0007
D	.3	370	Tan, turbid	56.2	.37	.0007
E	.4	520	Clear deep red	28.2	.24	.0008
F	.5	1340	Clear light red	73.0	1.00	.0014
G	.7	425	Colorless	17.0	0.10	.0006

Solution F, containing the highest percentage of copper, was selected for obtaining the copper protein in still higher state of purity. It was subjected to more fractional precipitation by means of varying concentrations of ammonium sulfate, following much the same procedure as that followed in obtaining the solutions described in Table I. The solutions thus obtained are described in Table II.

TABLE II

Soln.	Satn. amt. of $(NH_4)_2SO_4$ used	Vol., cc.	Color	Solids exclusive of salts, g.	Mg Cu	% Cu
H	0.6	1365	Deep red	68.60	0.64	0.0013
I	.7	510	Light red	3.10	.18	.0056
J	Saturated	320	Colorless	0.84	.10	.0120

Solution J, containing the highest percentage of copper, was selected for further purification of the copper protein. For this purpose 3 cc. of a 2.5% basic lead acetate solution was added to the 320 cc. of solution J. This operation was followed immediately by the addition of 30 cc. of cold acetone (cooled with solid carbon dioxide). The precipitate formed was removed by centrifugation and discarded since it contained no copper. To the remaining liquid 3 cc. more of the basic lead acetate solution was added followed by the addition of 30 cc. of cold acetone, the resulting precipitate removed by centrifugation and discarded. To the clear supernatant liquid, 30 cc. more of cold acetone was added and the precipitate M which was formed was separated by means of the centrifuge. Precipitate M, containing most of the copper, was suspended in 50 cc. of 0.1 M disodium phosphate, and allowed to stand for an hour, with intermittent stirring. Lead phosphate separated, was removed in the centrifuge, and the clear liquid thus obtained was dialyzed until free of phosphate. This dialyzed solution, M1, contained 0.23 g. of solids having 0.07 mg. or 0.03% of copper.

Further purification of the material contained in solution M1 was accomplished by adsorption to alumina at pH 6.8, followed by elution with 0.1 M disodium phosphate. After elution with 0.1 M disodium phosphate and dialysis, the resulting solution (solution O) was found to contain 0.14 g. of solids containing 0.06 mg. or 0.043% of copper.

Solution O was then treated dropwise with 2.5% basic lead acetate solution (about 2 cc.) at pH 7.5 until no further precipitate formed. The precipitate (P) was removed by centrifugation, and the clear supernatant liquid, containing no copper, discarded. The precipitate P was then suspended in 40 cc. of 0.1 M disodium phosphate and allowed to stand for three hours with intermittent stirring. The lead phosphate which had formed was removed in the centrifuge and the clear liquid dialyzed, yielding solution

P1. The latter contained 0.04 g. of solids, containing 0.034 mg. or 0.085% of copper.

The contents of solution P1 were then subjected to adsorption to alumina followed by elution by means of 0.1 M disodium phosphate. The clear solution thus obtained was then dialyzed for four days at 0° yielding a solution Q, which contained 0.0088 g. of solids (dry basis). This solid matter, on analysis, was found to contain 0.017 mg. or 0.19% of copper and 15% of nitrogen.

Determination of Copper.—The method used for the copper determinations was that of Warburg as modified by Warburg and Krebs.[13]

Determination of Nitrogen.—Two samples from solution Q, one equivalent to 2 and the other to 2.8 mg. of solids, were analyzed for nitrogen by the micro-Kjeldahl method. Both determinations gave values for nitrogen content equal to 15%.

To ensure no contamination by extraneous copper, all the samples used for copper determinations were dialyzed against distilled water which had been redistilled in glass vessels.

Dry weights of the solids were determined by the method described by Lutz and Nelson.[14]

The Copper in the Protein Is Non-ionic.—A freshly prepared milk protein fraction, containing in solution 540 mg. of solids (dry basis), which in turn had a copper content of 0.0185%, was divided into two equal portions, (I) and (II). To one aliquot, (I), was added 0.05 mg. of copper ion($CuSO_4$) thereby bringing the copper in its solid matter (the latter determined by evaporating a given amount of the solution) up to 0.037%. The two solutions, (I) and (II), were dialyzed separately at 0° for six days, at pH 6.5. At the end of each of the first three days, the solutions were removed from the dialyzing bags, and samples taken for copper determinations. The remaining solutions were again placed in fresh bags[15] and the dialysis
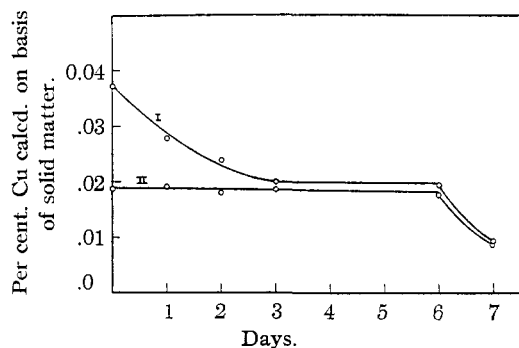

Fig. 1.

continued. As shown in Fig. 1, at the end of the three days of dialysis, the copper content of the solids in solution (I) had dropped to practically the same value (0.018%) as that of solution (II). On the other hand, the copper content of the solids in (II) remained at the original value (0.018%). In other words, the added ionic copper in solution (I) was lost in the dialysis while the copper belonging to the milk protein was not. From the end of the

(13) Warburg and Krebs, *Biochem. Z.*, **190**, 143 (1927).

(14) Lutz and Nelson, *J. Biol. Chem.*, **107**, 169 (1934).

(15) Visking sausage casing, made by the Visking Corporation, Chicago, was used in all dialyses.

third day up to the end of the sixth day of the dialysis, both solutions (I) and (II) lost no copper. Since most copper proteins lose copper in dilute acid solutions,[16] at the end of the sixth day both solutions were made pH 3.5 with hydrochloric acid, and under these conditions the copper belonging to the milk protein was lost in the dialysis.

The authors wish to express their thanks to the Borden Company for their generous supply of milk which made this study possible.

(16) Kubowitz, *Biochem. Z.*, **299**, 32 (1938).

Summary

A procedure is described for isolation of a copper bearing protein from cow's milk.

The copper in the protein is non-ionic and cannot be removed by dialysis at pH 6.5.

Up to the present, no chemical reactions have been found which are catalyzed by this milk protein.
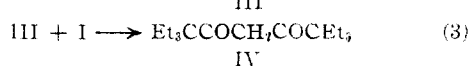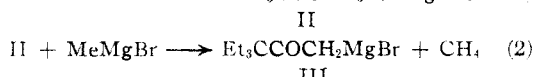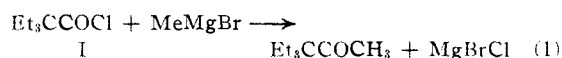

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY AND PHYSICS OF THE PENNSYLVANIA STATE COLLEGE]

Abnormal Grignard Reactions. XIV.[1] Sterically Hindered Aliphatic Carbonyl Compounds. IV. Methyl Triethylcarbinyl Ketone and its Bromomagnesium Enolate

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An attempt to prepare 2-methyl-3,3-diethyl-2-pentanol by the reaction of methylmagnesium bromide with triethylacetyl chloride was unsuccessful. The action of the Grignard reagent with the acid chloride caused the evolution of one-half mole of methane for each mole of Grignard reagent used. The liquid products were methyl triethylcarbinyl ketone and bis-triethylacetyl-methane.

$$Et_3CCOCl + MeMgBr \longrightarrow$$
$$I \qquad\qquad Et_3CCOCH_3 + MgBrCl \quad (1)$$
$$II$$
$$II + MeMgBr \longrightarrow Et_3CCOCH_2MgBr + CH_4 \quad (2)$$
$$III$$
$$III + I \longrightarrow Et_3CCOCH_2COCEt_3 \quad (3)$$
$$IV$$

Such a series of reactions is not new. Methyl-*t*-butylneopentylacetyl chloride[3] and dineopentylacetyl chloride[4] have both been found to give good yields of the corresponding methyl ketones with methylmagnesium bromide. These ketones give bromomagnesium enolates which react as true Grignard reagents.[3,4,5] Fuson and co-workers[6] had earlier shown that mesityl alkyl ketones give halomagnesium enolates which also react as true Grignard reagents.

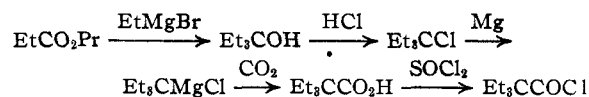Methyl triethylcarbinyl ketone gave 94% enolization and no addition when run in the

(1) XIII, Whitmore and Lester, THIS JOURNAL, **64**, 1251 (1942).
(2) Present address: Calco Chem. Div., American Cyanamide Co., Bound Brook, New Jersey.
(3) Whitmore and Randall, THIS JOURNAL, **64**, 1242 (1942).
(4) Whitmore and Lester, *ibid.*, **64**, 1247 (1942).
(5) Whitmore and Lester, *ibid.*, **64**, 1251 (1942).
(6) Fuson and co-workers, *ibid.*, **52**, 5036 (1930); **61**, 2362 (1939); *J. Org. Chem.*, **4**, 111 (1939).

Grignard machine[7] with methylmagnesium bromide. Thus, it is the lowest molecular weight ketone known which gives only enolization with the methyl Grignard reagent. The bromomagnesium enolate of methyl triethylcarbinyl ketone acts as a true Grignard reagent. Treatment of the enolate with carbon dioxide and formaldehyde gave the corresponding beta-keto acid and beta-ketol, respectively.

The few reactions studied indicate that the carbonyl of methyl triethylcarbinyl ketone is almost as sterically hindered as that of methyl methyl-*t*-butylneopentylcarbinyl ketone,[3] methyl dineopentylcarbinyl ketone[4] and acetomesitylene.[6] This is interesting when it is remembered that the reactions of pinacolone indicate only slight steric influence on the carbonyl. Thus, the remarkable difference in steric influence of the methyl and ethyl groups is clearly demonstrated.

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Experimental

The Grignard reagents for this work were prepared in the usual manner. All fractionations were done with the usual type of column[8] having 12–18 theoretical plates. The triethylacetyl chloride was prepared by standard reactions as follows:

$$EtCO_2Pr \xrightarrow{EtMgBr} Et_3COH \xrightarrow{HCl} Et_3CCl \xrightarrow{Mg}$$
$$Et_3CMgCl \xrightarrow{CO_2} Et_3CCO_2H \xrightarrow{SOCl_2} Et_3CCOCl$$

The chloride had b. p. 98° (65 mm.); $n^{20}D$ 1.4438.

(7) Kohler, Stone and Fuson, THIS JOURNAL, **49**, 3181 (1927).
(8) Whitmore and Lux, *ibid.*, **54**, 3451 (1932).