should be at the upper end of the range mentioned above. This would minimize the error in estimating bioavailability of iron from a simple ratio of response to dose which arises from failure of the true regression of response on dose to pass through the origin of the axes. Calculation of net hemoglobin gain requires only animal weights in addition to the hemoglobin measurements usually made in bioassays. The gain in precision of the estimate of iron availability would appear to justify the small increase in labor.

ACKNOWLEDGMENT

The author gratefully acknowledges the assistance of D. Landes, R. Stinchcomb, R. Mathews, and S. Donehoo.

LITERATURE CITED

Amine, E. K., Hegsted, D. M., *J. Nutr.* **101**, 927 (1971).
Amine, E. K., Hegsted, D. M., *J. Agric. Food Chem.* **22**, 470 (1974).
Amine, E. K., Hegsted, D. M., *J. Agric. Food Chem.* **23**, 204 (1975).
Amine, E. K., Neff, R., Hegsted, D. M., *J. Agric. Food Chem.* **20**, 246 (1972).
Anderson, T. A., Kim, I., Fomon, S. J., *Nutr. Metab.* **14**, 355 (1972).
Duncan, D. B., *Biometrics* **11**, 1 (1955).
Evelyn, K. A., Malloy, H. T., *J. Biol. Chem.* **126**, 655 (1938).
Finney, D. J., "Statistical Method in Biological Assay", Charles Griffin & Company, London, 1964.
Fritz, J. C., Pla, G. W., Harrison, B. N., Clark, G. A., *J. Assoc. Off. Anal. Chem.* **57**, 513 (1974).
Fritz, J. C., Pla, G. W., Harrison, B. N., Clark, G. A., *J. Assoc. Off. Anal. Chem.* **58**, 902 (1975).
Hegsted, D. M., Neff, R., Worcester, J., *J. Agric. Food Chem.* **16**, 190 (1968).
Mahoney, A. W., VanOrden, C. C., Hendricks, D. G., *Nutr. Metab.* **17**, 223 (1974).
Miller, J., *Cereal Chem.* **53**, 33 (1976a).
Miller, J., *Nutr. Metab.*, in press (1977).
Pla, G. W., Fritz, J. C., *J. Assoc. Off. Anal. Chem.* **53**, 791 (1970).
Pla, G. W., Fritz, J. C., *J. Assoc. Off. Anal. Chem.* **54**, 13, 490 (1971).
Ranhotra, G. S., Hepburn, F. N., Bradley, W. B., *Cereal Chem.* **48**, 177 (1971).
Rose, M. S., Vahlteich, E. M., *J. Biol. Chem.* **96**, 593 (1932).
Snedecor, G. W., "Statistical Methods", 4th ed, Iowa State College Press, Ames, Iowa, 1946.
Stookey, L. L., *Anal. Chem.* **42**, 779 (1970).
Williams, M. A., Chu, L-C., McIntosh, D. J., Hincenbergs, I., *J. Nutr.* **94**, 377 (1968).

Received for review May 27, 1976. Accepted September 2, 1976.

Binding of Mercury(II) Ion to Hen Egg White Lysozyme and Bovine Pancreatic Ribonuclease A

David C. Y. Ting and Eugene E. Schrier*

The binding of mercury(II) ion to hen egg white lysozyme and bovine pancreatic ribonuclease A was studied over a range of mercury(II) ion concentrations at pH 2.9 and 25 °C. An electrochemical method was employed involving the use of an iodide ion selective electrode which is responsive to mercury(II) ion. Only one class of binding sites was found for each protein. The number of binding sites is 19 ± 4 for lysozyme and 28 ± 4 for ribonuclease A. The binding constants are (5.52 ± 0.65) × 10^4 M^{-1} for lysozyme and (3.23 ± 0.28) × 10^4 M^{-1} for ribonuclease A. Each binding site may be comprised of two or more ligands with the mutual participation of side-chain carboxyl and amide groups and backbone peptide linkages.

The binding of mercury to thiol groups in various small molecules as well as in proteins has been extensively studied (Webb, 1966; Vallee and Ulmer, 1972). Mercury interactions with sulfur provide the basis for the toxicological effect of the metal in living systems.

The importance of Hg–S binding has led to a paucity of data pertaining to the interaction of Hg(II), i.e., Hg^{2+} ion, with proteins not containing –SH groups. Even in the one study of complexation of Hg(II) ion to groups on a protein other than –SH, an –SH containing protein, bovine serum albumin, was used. Bovine albumin contains from 0.50 to 0.75 –SH groups per mole (Hughes, 1947). At neutral pH, a complex containing one mercury(II) ion and 2 mol of albumin has been demonstrated and is known as mercaptalbumin. The mercury(II) ion is presumed to link the protein molecules through their thiol groups. At pH values below 4, the situation is strikingly different. Perkins (1961) found that 85 mol of Hg(II) ion is bound per mole of bovine serum albumin. He also studied the binding of mercury(II) ion to chemically modified albumins. He found that the binding of mercury(II) ion increased when amine groups which are positively charged at the pH of interest are modified by amide formation so as to reduce the charge. Perkins concluded from these experiments that the mercury(II) ions are probably bound to carboxylate groups. An unfortunate ambiguity was present in his experiment, however. He utilized sodium acetate–acetic acid as his buffer medium. Mercury(II) ion is known to bind to acetate ion (Webb, 1966). The extent of mediation of the buffer ion in the binding of mercury(II) to the protein as well as the intrinsic equilibrium constant for the interaction are, therefore, unknown.

The present investigation was initiated to characterize quantitatively the binding of mercury(II) ion to two proteins which do not contain sulfhydryl groups. We also hoped to make inferences regarding the nature of the binding site or sites for this ion.

MATERIALS AND METHODS

Proteins. Hen egg-white lysozyme (type I) and bovine ribonuclease A (type XIIA) were both purchased from Sigma Chemical Co. The water content of each protein was determined by heating to constant weight at 120 °C. Correction for this was made in all calculations. Two

Department of Chemistry, State University of New York at Binghamton, Binghamton, New York 13901.

different molar concentrations of lysozyme were employed during this study while one concentration of ribonuclease A was used.

Reagents. A stock solution of mercury(II) nitrate was prepared by dissolving a weighed portion of reagent grade crystals in an aqueous solution of KNO_3 and HNO_3. More dilute mercury(II) nitrate solutions (to 10^{-6} M) were prepared from this stock solution. In all cases, these solutions were pH 2.9 and contained 0.1 M KNO_3 as the background electrolyte.

Apparatus. A pH meter (Model 801, Orion Research Inc.) was employed in conjunction with an iodide specific ion electrode (Model 94-53, Orion Research Inc.) and a double junction reference electrode (Model 90-02, Orion Research Inc.) with 1 M KNO_3 solution in the outer chamber. It has been found empirically that this electrode responds to mercury(II) ion down to about 10^{-8} M (Orion, 1970). While the mechanism is not established, it appears to involve a reaction of mercury(II) ion with the silver iodide of the membrane surface to release silver ion. The electrode then senses the silver ion released. Since a monovalent ion is detected, potential vs. log Hg(II) ion concentration plots exhibit a monovalent slope in spite of the fact that mercury(II) ion is divalent.

Determination of Mercury(II) Ion Interaction with Proteins. One hundred milliliters of a solution of mercury(II) nitrate was placed in a beaker which was contained in an oil bath controlled at 25.0 ± 0.1 °C. The electrodes were inserted and the solution was allowed to equilibrate by continuous stirring using a submersible magnetic stirrer. During this time, the potential was recorded at 5-min intervals. When the potential became steady, i.e., drift generally less than 0.1 mV in a 15-min period, 1 ml of an appropriate protein solution was added and the potential was followed again until it was steady. After each sequence, the iodide electrode was placed into a solution of 0.001 M sodium iodide for 10 s and rinsed in distilled water. A higher concentration of mercury(II) ion was employed in the next run. Runs were continued at increasing mercury(II) ion concentrations until the difference between the potential reading before and after the addition of mercury(II) ion was too small to be meaningful.

Potential measurements were reproducible to ±0.1 mV. Although there were no other significant uncertainties in measurement, chemical problems in the systems to be described below limited the repeatibility in replicate runs to ±5%.

RESULTS

General Observations Regarding the Measurements. Although the electrochemical procedure for following the binding reaction appeared to yield good results, the fact that precipitates appeared in the mercury(II) ion–protein system at mercury(II) ion concentrations above 1.5×10^{-5} M reduced the overall precision of the results. The general level of precision still is satisfactory when compared with other studies carried out using electrodes. Previous studies by Tanford and Epstein (1954) and by Riddiford and Scheraga (1962) have indicated that binding constants are not affected by precipitation as long as there is free passage of the binding ion from the bulk solution to the binding sites. The absence of abrupt changes in the slope of the data to be presented suggests that this condition was fulfilled in these experiments.

Determination of Binding Constants from the Experimental Data. The data obtained are potentials generated by the mercury(II) ion in the presence and absence of protein. We desire first to calculate the concentration of free mercury(II) ion in equilibrium with mercury(II) ion bound to the protein. Let E_1 = the measured potential before the addition of the protein to the solution containing Hg^{2+} ion, E_2 = the measured potential of the solution after the addition of the protein to the solution containing Hg^{2+} ion, $(a_{Hg^{2+}})_1$ = the activity of the Hg^{2+} ion before adding the protein, and $(a_{Hg^{2+}})_2$ = the activity of the Hg^{2+} ion after adding the protein. Then applying the Nernst equation to each solution:

$$E_1 = E' + 2.303(RT/F) \log (a_{Hg^{2+}})_1 \quad (1)$$

$$E_2 = E' + 2.303(RT/F) \log (a_{Hg^{2+}})_2 \quad (2)$$

In eq 2 we assume that E' is not modified by the presence of the protein, i.e., that the only function of the protein is to take up Hg^{2+} ions which are then not sensed by the electrode. Subtracting eq 1 from eq 2 we obtain eq 3:

$$E_2 - E_1 = 2.303(RT/F) \log \frac{(a_{Hg^{2+}})_2}{(a_{Hg^{2+}})_1}$$

$$= 2.303(RT/F) \log \frac{(C_{Hg^{2+}})_2 (y_{Hg^{2+}})_2}{(C_{Hg^{2+}})_1 (y_{Hg^{2+}})_1} \quad (3)$$

where the $y_{Hg^{2+}}$ represent the molar activity coefficients. In order to calculate $(C_{Hg^{2+}})_2$ some assumptions must be made regarding the values of $(y_{Hg^{2+}})_2$ and $(y_{Hg^{2+}})_1$. When the experiments are conducted at a constant ionic strength, $(y_{Hg^{2+}})_2$ may be taken as equal to $(y_{Hg^{2+}})_1$ to a good approximation (Rossotti and Rossotti, 1961). Then $(C_{Hg^{2+}})_2$ may be obtained.

We next consider the reaction, P + A $\rightleftharpoons$ PA, where P represents a protein molecule, A is a small molecule or ion (not designated with a charge for simplicity), and PA is the protein–small molecule complex. We define $\bar{\nu}$ as the mean number of moles of A bound to 1 mol of protein. In the case of mercury(II) ion binding studied here, a given value of $\bar{\nu}$ can be obtained by subtracting the concentration of free mercury(II) ion, $(C_{Hg^{2+}})_2$, obtained in the electrode measurements described above, from the initial mercury(II) ion concentration present when the solution was made up. The difference in concentration is the number of moles per liter of mercury(II) ion bound to the protein. If this is divided by the protein concentration in moles per liter, the result is the moles of mercury(II) ion bound per mole of protein.

Scatchard (1949) showed that the number of binding sites, n, and the binding constant, K, could be obtained from the equation:

$$\bar{\nu}/[A] = K(n - \bar{\nu}) \quad (4)$$

In practice, a plot of $\bar{\nu}/[A]$ vs. $\bar{\nu}$ is constructed. If it is a straight line, then the slope is $-K$ and the intercept is Kn so that n can be determined. More complex situations can be handled as necessary.

Derived Results for the Binding of Mercury(II) Ion to Lysozyme and Ribonuclease A. The experimental results and calculated parameters for mercury(II) ion binding are given for lysozyme in Table I and for ribonuclease A in Table II. Plots of $\bar{\nu}/(C_{Hg^{2+}})_2 \times 10^5$ M vs. $\bar{\nu}$ are shown for lysozyme and for ribonuclease A in Figures 1 and 2. The lines drawn are determined by a least-squares treatment of the data. Because of the lower precision of the data in the region in which precipitate formed, more trials were made there leading to an overabundance of points in the region. In order not to overemphasize this factor in the least-squares treatment, an average value for all these points was calculated and considered to be a simple data point in the least-squares routine. While this method of data treatment did produce

Table I. Experimental Data and Calculated Parameters for the Binding of Mercury(II) Ion to Lysozyme at 25 °C

Initial Hg(II) ion concn, $(C_{Hg^{2+}})_1 \times 10^5$ M	Potential diff, $-\Delta E$, mV	Free Hg(II) ion concn (calcd), $(C_{Hg^{2+}})_2 \times 10^5$ M	$\bar{\nu}$	$\bar{\nu}/(C_{Hg^{2+}})_2 \times 10^5$ M
		Lysozyme Concn = 3.74×10^{-6} M		
7.89	20.5	3.35	12.1	3.627
7.89	20.5	3.35	12.1	3.627
10.3	18.0	4.85	14.6	3.002
10.3	16.0	5.28	13.4	2.547
10.3	16.5	5.17	13.7	2.657
12.9	14.0	7.19	15.3	2.128
12.9	14.7	6.98	15.8	2.270
17.2	9.9	11.4	15.6	1.371
17.2	10.0	11.3	15.7	1.388
21.5	7.4	15.8	15.3	0.970
21.5	7.8	15.5	16.0	1.031
21.5	7.5	15.7	15.5	0.985
21.5	7.7	15.6	15.8	1.016
37.4	3.8	31.9	14.7	0.461
37.4	4.2	31.4	16.1	0.513
64.4	2.4	58.3	16.4	0.282
64.4	2.2	58.7	15.1	0.258
64.4	2.5	58.0	17.1	0.295
108.5	1.4	102	16.5	0.161
140	1.5	131	22.8	0.173
215	1.0	206	23.5	0.114
		Lysozyme Concn = 2.81×10^{-6} M		
6.03	19.2	2.70	11.8	4.381
6.03	19.9	2.62	12.1	4.617
15.0	10.7	9.59	19.3	2.007
15.0	10.1	9.83	18.4	1.869
30.2	5.7	23.8	22.8	0.957
30.2	5.6	23.9	22.4	0.939
60.3	1.8	55.9	15.6	0.278
60.3	2.1	55.2	18.0	0.326
120.6	1.4	113.7	24.2	0.214

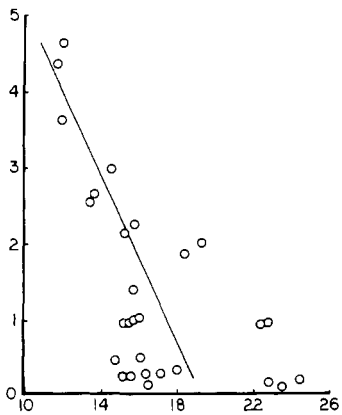

Figure 1. The quantity, $\bar{\nu}/[(C_{Hg^{2+}})_2 \times 10^5$ M], plotted against $\bar{\nu}$ for mercury(II) ion binding to lysozyme at 25 °C.

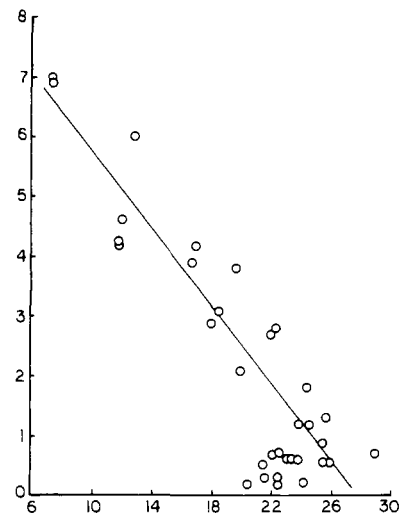

Figure 2. The quantity, $\bar{\nu}/[(C_{Hg^{2+}})_2 \times 10^5$ M], plotted against $\bar{\nu}$ for mercury(II) ion binding to ribonuclease A at 25 °C.

a small effect on the derived parameters (given below) for mercury(II) ion binding to lysozyme, there was no effect for ribonuclease A. In the case of lysozyme, however, the line calculated using this procedure fit the early points much better than if each point was utilized as a separate value in the least-squares procedure.

The slope and intercept of the plots were used to calculate K and n by means of eq 4. The results of the calculation are shown in Table III. The uncertainties quoted are standard deviations generated by the statistical treatment.

DISCUSSION

The number of binding sites, n, and the equilibrium constant, K, are sufficiently similar for both proteins to allow the hypothesis that the mechanism of binding is the same in both cases. In addition, the simple nature of the Scatchard plots suggests only one class of binding sites.

Examination of the amino acid composition for each of these proteins given in Table IV indicates no single likely residue for binding which is present in the number required for each protein. A combination of different classes of residues is possible but would require either that (1) there be multiphasic behavior of the binding curve or (2) the different residues have very similar binding constants with Hg(II) ion. The first possibility is pretty much ruled out by the experimental results although curvature in Figures 1 and 2 could be obscured by the scatter of the data. Let us explore the second possibility in more detail.

A hypothetical scheme which would provide the correct number of sites is to combine the carboxylic acid groups and the carboxylic acid amides and consider the combi-

Table II. Experimental Data and Calculated Parameters for the Binding of Mercury(II) Ion to Ribonuclease A at 25 °C

Initial Hg(II) ion concn, $(C_{Hg^{2+}})_1 \times 10^5$ M	Potential diff, $-\Delta E$, mV	Free Hg(II) ion concn (calcd), $(C_{Hg^{2+}})_2 \times 10^5$ M	$\bar{\nu}$	$\bar{\nu}/(C_{Hg^{2+}})_2 \times 10^5$ M
Ribonuclease Concn = 6.75×10^{-6} M				
6.03	41.5	1.06	7.4	6.916
6.03	41.6	1.06	7.3	6.951
10.8	38.6	2.15	12.8	5.957
10.8	33.9	2.62	12.1	4.630
10.8	32.3	2.80	11.9	4.235
10.8	32.4	2.79	11.9	4.259
15.5	32.0	4.07	16.9	4.164
15.5	30.9	4.26	16.7	3.910
18.1	25.6	6.21	18.0	2.894
18.1	30.0	5.17	19.5	3.784
18.1	25.6	2.95	18.4	3.083
22.9	21.1	9.52	19.8	2.083
22.9	25.0	8.05	22.0	2.732
22.9	25.5	7.89	22.2	2.821
22.9	25.4	7.92	22.2	2.803
30.2	18.8	13.8	24.4	1.770
30.2	18.9	13.7	24.4	1.783
36.2	15.2	19.2	25.7	1.341
36.2	13.7	20.4	23.8	1.168
36.2	14.2	20.0	24.5	1.225
45.1	9.6	30.2	22.1	0.732
45.1	9.8	29.9	22.5	0.750
45.2	11.1	28.4	25.4	0.892
54.4	7.2	40.3	21.4	0.531
54.4	8.0	38.9	23.4	0.600
54.4	7.9	39.1	23.1	0.591
54.4	8.2	38.6	23.9	0.618
60.3	8.2	42.8	25.9	0.609
60.3	9.3	40.8	28.8	0.704
60.3	8.0	43.2	25.4	0.588
90.0	4.4	74.9	22.4	0.299
90.0	4.2	75.7	21.5	0.284
120.6	2.9	106.8	20.4	0.191
120.6	3.2	105.5	22.4	0.212
120.6	3.4	104.6	24.1	0.231

Table III. Equilibrium Constant and Number of Binding Sites for the Binding of Mercury(II) Ion to Lysozyme and Ribonuclease A at 25 °C

	n	K
Lysozyme	19.2 ± 3.8	$(5.52 \pm 0.65) \times 10^4$ M^{-1}
Ribonuclease A	27.9 ± 3.7	$(3.23 \pm 0.28) \times 10^4$ M^{-1}

nation as the single class of sites for each protein. For lysozyme the number is 27 while for ribonuclease A it is 28. In both cases, we have included the C-terminal carboxylic acid group. These numbers are of the correct magnitude. However, we require some evidence that the intrinsic binding constants are similar enough to be treated as one class.

Let us consider the carboxyl groups first. According to the titration curves (Tanford and Wagner, 1954; Tanford et al., 1955), about 25% of the carboxyls are ionized for each protein at pH 2.9. Using an equation given by Breslow (1973):

$$K_m' = \frac{K_m}{1 + K_h[H^+]} \quad (5)$$

where K_m is the association constant of the metal with the fully deprotonated carboxyl group and K_h is the carboxylate ion–proton association constant, we can calculate K_m' which is the metal–carboxylate association constant under conditions in which the carboxylate ion is partially protonated. We take $K_h = 2 \times 10^4$ rather than the normal value of 5×10^4 to correct for the anomalous titration behavior of both proteins in the acid region. The value of K_m is 1×10^4 (Webb, 1966). Then K_m' is 3×10^2 and log $K_m' = 2.48$.

Table IV. Amino Acid Composition of Lysozyme and Ribonuclease A[a]

Amino acid	Lysozyme, residues/mol	Ribonuclease A, residues/mol
Alanine	12	12
Arginine	11	4
Asparagine	13	11
Aspartic acid	8	4
Half-cystine	8	8
Glutamic acid	2	6
Glutamine	3	6
Glycine	12	3
Histidine	1	4
Isoleucine	6	3
Leucine	8	2
Lysine	6	10
Methionine	2	4
Phenylalanine	3	3
Proline	2	4
Serine	10	15
Threonine	7	10
Tryptophan	6	0
Tyrosine	3	6
Valine	6	9
Total	129	124

[a] Dayhoff and Eck (1968).

The calculation for the association constant of Hg(II) ion with an amide group is more involved. From the data of Gould and Sutton (1970) we can obtain log K_6 for reaction 6 as 15.69. Combining this with the log K_7 for

$$HgCl_2 + 2(C(=O)NH^-) \rightarrow Hg(HNC(=O))_2 + 2Cl^- \quad (6)$$

reaction 7 (Webb, 1966), which is 13.22, we obtain log K_8

$$Hg^{2+} + 2Cl^- \rightarrow HgCl_2 \quad (7)$$

for reaction 8 as 28.91. The log K_9 for reaction 9 (Gould

$$Hg^{2+} + 2(C(=O)NH^-) \rightarrow Hg(HNC(=O))_2 \quad (8)$$

and Sutton, 1970) is 30.2, so that subtracting eq 9 from

$$2(C(=O)NH^-) + 2H^+ \rightarrow 2(C(=O)NH_2) \quad (9)$$

eq 8 we finally obtain:

$$Hg^{2+} + 2(C(=O)NH_2) \rightarrow Hg(HNC(=O))_2 + 2H^+ \quad (10)$$

and log $K_{10} = -1.3$. For 1 mol of amide bound to Hg(II) we can take the square root as a good approximation and have log $(K_{10})^{1/2} = -0.65$. The major uncertainty in this result comes from the use of log K_9 which might vary by one unit to either side of the reported value.

This calculation suggests that the two types of groups, i.e. COOH and $CONH_2$, should be distinguishable by Scatchard analysis since the ratio of the K values is about 1000:1. No distinction is apparent from the experimental data. In addition, neither site by itself yields a K which is large enough to compare with the experimental values of $>10^4$. Indeed, even larger intrinsic constants for the association of Hg(II) with the proteins are to be expected since the charge on both proteins is high at this pH, +18 for lysozyme and +14 for ribonuclease A.

We conclude that the carboxylic acid and carboxylic acid amide groups cannot together provide a single class of sites for the binding of Hg(II) ion to either protein. It is known from the study of model systems (Kamenar and Grdenic, 1969) that mercury(II) ion forms two strong bonds with ligands and may also form one or more weaker ones. Therefore, a likely possibility is that the binding sites for Hg(II) ion are not monofunctional, i.e., consisting of a single carboxylic acid or a single amide group. Instead, each site may be comprised of two or more ligands. These may be the aforementioned side-chain carboxyl and amide groups and backbone peptide linkages. Indeed, even the aromatic side-chain groups could participate since benzene is known to be mercurated (Westheimer et al., 1947) in aqueous nitric acid solutions at slightly more elevated temperatures. Steric arrangements of the ligands consistent with the primary structure of the protein will determine which groups on the protein are involved in the binding. Further work is required to better characterize the nature of these sites.

LITERATURE CITED

Breslow, E., in "Inorganic Biochemistry", Eichhorn, G. I., Ed., Elsevier Scientific Publishing Co., New York, N.Y., 1973, p 227.
Dayhoff, M. O., Eck, R. V., "Atlas of Protein Sequence and Structure", National Biomedical Research Foundation, 1968.
Gould, R. O., Sutton, H. M., *J. Chem. Soc. A*, 1184 (1970).
Hughes, W. L., Jr., *J. Am. Chem. Soc.* **69**, 1836 (1947).
Kamenar, B., Grdenic, D., *Inorg. Chem. Acta* **3**, 25 (1969).
Orion Research Incorporated Newsletter, Vol. 2, 1970, p 41.
Perkins, D. J., *Biochem. J.* **80**, 668 (1961).
Riddiford, L., Scheraga, H. A., *Biochemistry* **1**, 95 (1962).
Rossotti, F. J. C., Rossotti, H., "The Determination of Stability Constants", McGraw-Hill, New York, N.Y., 1961, p 20.
Scatchard, G., *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).
Tanford, C., Epstein, J., *J. Am. Chem. Soc.* **76**, 2163 (1954).
Tanford, C., Hauenstein, J. D., Rands, D. G., *J. Am. Chem. Soc.* **77**, 6409 (1955).
Tanford, C., Wagner, M., *J. Am. Chem. Soc.* **76**, 3331 (1954).
Vallee, B. L., Ulmer, D. D., *Annu. Rev. Biochem.* **41**, 91 (1972).
Webb, J. L., "Enzyme and Metabolic Inhibitors", Vol. 2, Academic Press, New York, N.Y., 1966, p 729.
Westheimer, F. H., Segel, E., Schramm, R., *J. Am. Chem. Soc.* **69**, 773 (1947).

Received for review March 3, 1976. Accepted September 30, 1976. Taken in part from the thesis of David C. Y. Ting submitted to S.U.N.Y. of Binghamton in partial fulfillment of the Master of Arts Degree. This work was supported in part by Grant No. GM 11762 from the Institute of General Medical Sciences, U.S. Public Health Service.

Preparation of Chromium-Containing Material of Glucose Tolerance Factor Activity from Brewer's Yeast Extracts and by Synthesis

Edward W. Toepfer, Walter Mertz,* Marilyn M. Polansky, Edward E. Roginski, and Wayne R. Wolf

When Brewer's yeast was extracted with dilute alcohol and purified by ion exchange chromatography the resulting preparations were shown to have glucose tolerance factor (GTF) activity. They potentiated the action of insulin on the glucose oxidation of chromium-deficient rat adipose tissue in vitro. Such preparations were found to contain chromium, nicotinic acid, glycine, glutamic acid, and cysteine. Reacting trivalent chromium with these ligands in vitro yielded a mixture of chromium complexes which exhibited GTF properties, similar to the material separated from Brewer's yeast.

The glucose tolerance factor (GTF) contains the essential trace element, chromium (Mertz, 1969). This compound (or group of compounds) occurs in Brewer's yeast and other foods (Toepfer et al., 1973); its exact structure is yet unknown. Chromium as part of GTF is more potent in chromium-deficient animals than are simple chromium compounds, such as tetra- or hexaaquo complexes. The best known function of GTF is the potentiation of the action of insulin on chromium-deficient tissue (Mertz and Roginski, 1971). Not all chromium in biological materials has GTF activity, as the latter is not significantly correlated to the *total* chromium content of different materials. There is, however, a significant correlation between biological function and that part of the total chromium which is extractable by 50% ethanol (Toepfer et al., 1973). Brewer's yeast was found to be outstanding among all foods tested in that approximately one-third of its high chromium content was in this form;

Nutrition Institute, U.S. Department of Agriculture, Agricultural Research Service, Beltsville, Maryland 20705.