Study of Experimental Conditions for Most Reliable Estimates of Relative Biological Value of Iron in Bread

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The purpose of the study was to determine dietary iron concentrations and length of hemoglobin regeneration periods needed to give reliable estimates of the relative biological value (RBV) of bread iron. Anemic albino rats were fed diets supplemented with 6, 12, and 18 mg/kg iron supplied as ferrous sulfate or white wheat bread for 3, 7, and 11 days. The best dose–response correlations were obtained with total iron intakes of 1.5 to 4 mg for periods of 7 or 11 days. Net hemoglobin gain was a better measure of response than final hemoglobin concentration or change in concentration.

Bioavailability of iron in test sources was assessed by measuring regeneration of hemoglobin in anemic rats as long as 45 years ago (Rose and Vahlteich, 1932). With the development of sensitive colorimetric and atomic absorption methods for analysis of low concentrations of iron in foodstuffs, the more expensive and time-consuming bioassay was largely abandoned. Recent indications of the prevalence of iron-deficiency anemia in some segments of the population have created concern about the adequacy of dietary iron supply. Proposals to increase the level of iron fortification in some foods and to fortify more foods have been advanced. These concerns have spurred renewed interest in the biological availability of native iron in foods and of the iron in preparations used for fortification.

Pla and Fritz (1970) proposed a hemoglobin repletion test which was adopted (Pla and Fritz, 1971) for bioassay of iron. Attempts to standardize the test have included, among other factors, investigations of various methods of comparing data from test substances with those from the standard, ferrous sulfate. In the original procedure relative biological value (RBV) of iron was estimated by a graphic comparison of test substances fed at one level with a curve plotted from data obtained from ferrous sulfate fed at several different levels. Change in hemoglobin concentration of the blood during the regeneration period was the parameter evaluated. Later refinements in methodology have resulted in recommendations to compare data by the parallel-lines technique when both test and standard preparations are supplied at several dietary concentrations (Fritz et al., 1974). Final hemoglobin concentration was reported (Fritz et al., 1975) to be as effective as change in hemoglobin during the regeneration period as a criterion of response to iron supplements.

The slope–ratio test had been adjudged the best bioassay for estimating relative nutritive value of various protein sources (Hegsted et al., 1968). This same statistical program was used to evaluate biological availability of iron in different diets and foods (Amine and Hegsted, 1971, 1974). These authors (1974) pointed out that most dose–response curves are sigmoidal when studied over a wide range of doses and whether a parallel line or slope–ratio analysis is most appropriate depends upon the range of doses selected.

Using animal weight and hemoglobin concentration data obtained at the beginning and end of experimental periods, Ranhotra et al. (1971) calculated total gain in hemoglobin per unit of iron intake. These figures were used as a measure of iron availability from bread fortified with several different commercial enrichment preparations. Anderson et al. (1972) also evaluated several iron sources as enrichment for cereal–milk diets. They calculated the percent of dietary iron that could be accounted for by the increase in hemoglobin iron during the feeding period. Similarly, hemoglobin iron gain per unit of iron intake was employed by Mahoney et al. (1974) as a basis for comparing the efficiency of extraction of iron from various foods. In their study ferrous sulfate iron had an efficiency of 51%. They noted that data reported by five other laboratories showed similar conversion efficiency ratios for ferrous sulfate over a range of iron intake from 0.12 to 0.5 mg of Fe/day.

The hemoglobin repletion test originally proposed by Pla and Fritz (1970) for estimating bioavailability of iron specified a repletion period of 2 weeks and a 2-week regeneration period was used in subsequent studies in that laboratory (Fritz et al., 1974, 1975). However, these reports contain no data to substantiate the suitability of the time period selected. Other laboratories have reported data on animals repleted for periods ranging from 10 to 30 days. Ranhotra et al. (1971) indicated that estimates of relative availability of iron from several enrichment sources differed when calculated after 30 days' regeneration rather than after a 15-day period of recuperation. A more nearly linear response of hemoglobin regeneration to iron intake from ferrous sulfate at the dietary levels recommended (Fritz et al., 1974) was obtained in our laboratory by shortening the repletion period to 11 days (Miller, 1977).

Hematocrit had been suggested (Amine et al., 1972) as a possible alternate to hemoglobin as the parameter of response for estimation of bioavailability of iron. However, Amine and Hegsted later reported (1975) that substantial differences in potency for some diets were found when hematocrit rather than hemoglobin was used as the measure of response. Anderson et al. (1972) also noted a difference in response of hemoglobin and hematocrit to dietary treatments. Estimates of the relative biological value (RBV) of iron in bread were found to depend upon the hemotological parameter used (Miller, 1976a).

The shape of the dose–response curve obtained in a particular bioassay will depend on the range of total intake of the limiting nutrient. Since total intake is a function not only of dose level but also of duration of the feeding period, both level and time should be considered in establishing an analytical protocol. The study reported here was designed to establish an optimum range of total iron intake for comparing utilization of iron from wheat bread

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Table I. Total Iron Intake and Initial and Final Weights of Iron Deficient Rats Fed Regeneration Diets with Iron Supplements from Ferrous Sulfate or White Wheat Bread

Fe added		Total Fe,	Total Fe intake, mg			Initial wt, g			Final wt, g		
Source	mg/kg	mg/kg	3[a]	7	11	3	7	11	3	7	11
		5.7	0.27	0.56	0.96	153	159	166	171	184	215
$FeSO_4$	6	11.8	0.61	1.32	2.05	159	166	162	182	205	226
	12	18.9	0.98	2.50	3.87	165	174	167	188	228	239
	18	23.8	1.42	3.10	5.27	161	164	165	189	210	252
Bread	6	10.7	0.50	1.15	1.94	156	154	169	174	189	232
	12	15.6	0.84	1.84	2.92	176	167	165	205	213	241
	18	20.6	1.03	2.37	3.92	150	154	154	174	200	227

[a] Number of days regeneration diets were fed.

with that of iron from ferrous sulfate by anemic rats. In addition, several methods of relating response to intake were compared.

MATERIALS AND METHODS

Loaf bread made of white wheat flour was obtained from a local grocery store. The bread was oven dried at 60 °C and ground to pass a 20-mesh screen. The dried, ground bread contained 2.00% nitrogen by Kjeldahl analysis and 35.3 μg of iron per gram by the analytical procedure described below. Ingredients of the basal diet were (g/kg): casein, 143; dextrin, 450; L-methionine, 2; salt mixture [Williams and Briggs, modified, with ferric citrate omitted (Williams et al., 1968)], 35; vitamin mixture (formulated according to Vitamin Diet Fortification Mixture, ICN Nutritional Biochemicals), 22; soybean oil, 50; sucrose, 283; cellulose, 15. This basal diet contained 5.7 mg of Fe/kg. For the standard regeneration diets, ferrous sulfate triturated in powdered sugar was added to the basal diet at the expense of crystalline sucrose. Regeneration diets containing bread were prepared by substituting ground bread in the basal diet for casein and dextrin in proportion to the protein (N × 6.25) and nonprotein portions, respectively, of the bread.

Weanling male Sprague-Dawley rats (Charles River Breeding Laboratories) were housed individually in stainless steel cages with wire mesh floors. Food and deionized water were provided ad libitum. Spilled, unsoiled feed was returned to the feed cup each day and feed intake was measured three times per week. The basal diet was fed for 24 days. At this time the hemoglobin concentration, measured in blood obtained by amputating the tip of the tail, averaged 4.99%. Six animals were allotted to each of 21 groups in such a manner that the mean and standard deviation of hemoglobin concentration were similar for all groups. Three groups of rats were maintained on the basal diet and three groups were fed each of the regeneration diets. The control regeneration diets contained ferrous sulfate to supply 6, 12, and 18 mg of Fe/kg and the test diets contained 16.7, 33.3, and 50% bread which furnished 5.9, 11.8, and 17.6 mg of Fe/kg, respectively. One group of animals from each dietary treatment was sacrified after being fed the regeneration diets for 3 days, one group after 7 days, and the final group after 11 days.

The animals were anesthetized with sodium pentobarbital and blood was obtained in a heparinized syringe by cardiac puncture. Hemoglobin was determined by the cyanmethemoglobin method described by Evelyn and Malloy (1938), packed cell volume by microcapillary centrifugation, and red blood cells were counted using a General Scientific counter. Bread and diet samples were digested in nitric, perchloric, and sulfuric acids. Iron in these samples was reduced with hydroxylamine hydrochloride, chelated with ferrozine (Stookey, 1970), and measured colorimetrically.

Change in hemoglobin was expressed as the difference between hemoglobin concentration (g/100 ml) in the blood at the end and the beginning of the regeneration period. Total hemoglobin and hemoglobin iron were calculated on the assumption that 7% of the body weight is blood volume and that hemoglobin contains 0.34% iron. Net hemoglobin iron gain was estimated as the difference between total hemoglobin iron at the end and beginning of the regeneration period. Total iron intake for each animal was calculated from its food intake and analyzed values for iron content of the diets. Thus, the values used and reported include iron present in the dietary ingredients and that incorporated during mixing as well as that added as ferrous sulfate or bread iron.

Data from individual animals were used to calculate regression of response on total iron intake for each source of supplemental iron and for each discrete regeneration time period. The regression parameters for all measures of response were calculated by standard procedures with no assumption about the point of intersection of the regression line with the axes (Snedecor, 1946). In the particular case of net hemoglobin iron gain, a regression coefficient was also calculated assuming that the line intersected the origin of the axes. RBV of iron in bread compared to that of ferrous sulfate was calculated from ratios of regression coefficients (Finney, 1964). Analysis of variance (Snedecor, 1946) and Duncan's multiple range test (Duncan, 1955) were used where appropriate.

RESULTS AND DISCUSSION

Mean values for the basic data obtained on iron intake and animal weights (Table I) and blood values (Table II) are shown for comparative purposes. Parameters of the regression equations (Table III) indicate that for the levels of dietary iron concentration used in this experiment the slope–ratio test was a valid measure of relative potency of the iron in bread. The appropriate comparisons are between each pair of equations relating a hematological variable to iron intake for one time period. The line calculated from data obtained with rats fed diets containing bread intercepted the ordinate at approximately the same point as that from animals fed diets supplemented with ferrous sulfate.

The correlation coefficients (Table III) support several observations concerning the relationship of the blood parameters to iron intake during the regeneration period. Firstly, these coefficients were lower for data obtained from animals fed the bread diets than for those from rats provided with diets containing ferrous sulfate. Moreover, the dose–response correlations were improved by increasing the recuperative period from 3 to 11 days. Thus, in this experiment, a high correlation between intake and response was obtained only when iron intake ranged from about 1.0 to 5 mg. Since the available iron of bread is approximately 50% of the total, bread content of these diets would have to be increased to obtain the same

Table II. Blood Parameters of Iron Deficient Rats after Regeneration with Diets Containing Iron Supplements from Ferrous Sulfate or White Wheat Bread

Fe added		Total Fe,	Hemoglobin, g/100 ml			Packed cell vol, %			Red blood cell count, millions/μl		
Source	mg/kg	mg/kg	3[a]	7	11	3	7	11	3	7	11
		5.7	4.29	4.55	4.38	22.1	24.3	23.4	3.33	3.70	3.37
$FeSO_4$	6	11.8	4.93	5.58	5.89	24.8	28.3	30.9	3.68	4.34	4.68
	12	18.9	5.59	7.11	7.94	27.6	33.8	38.2	4.13	5.08	5.50
	18	23.8	6.57	7.78	10.03	31.4	38.2	43.7	4.30	5.26	5.92
Bread	6	10.7	4.62	4.85	4.70	23.9	25.8	25.2	3.64	3.66	3.69
	12	15.6	4.74	5.04	5.44	23.0	25.8	28.2	3.50	3.95	4.29
	18	20.6	4.82	5.51	6.15	24.8	28.7	31.8	3.62	4.25	4.74

[a] Number of days regeneration diets were fed.

Table III. Correlation and Regression Parameters for Several Measures of Hematological Response to Iron Intake by Anemic Rats[a]

	Final Hb, g/100 ml			Change in Hb, g/100 ml			Net gain in Hb-Fe, mg			Packed cell vol, %			Red cell count, millions		
	3[b]	7	11	3	7	11	3	7	11	3	7	11	3	7	11
							Ferrous Sulfate								
Corr coeff	0.65	0.74	0.92	0.64	0.88	0.85	0.76	0.94	0.94	0.58	0.82	0.91	0.55	0.56	0.80
Regression coeff	1.66	1.04	1.21	1.47	0.93	1.17	0.799	0.666	0.826	6.47	4.53	3.76	0.656	0.408	0.376
y intercept	4.03	4.42	3.45	−0.69	−0.27	−1.38	−0.156	−0.037	−0.483	21.4	23.0	23.6	3.36	3.96	3.06
							Bread								
Corr coeff	0.31	0.38	0.47	0.20	0.62	0.77	0.55	0.90	0.85	0.28	0.30	0.51	0.12	0.13	0.48
Regression coeff	1.02	0.51	0.55	0.27	0.47	0.62	0.289	0.394	0.428	3.83	2.02	2.43	0.273	0.120	0.393
y intercept	3.93	4.22	3.82	−0.55	−0.69	−1.39	−0.085	−0.143	−0.190	20.9	23.2	21.3	3.37	3.88	3.09
RBV	0.61	0.49	0.45	0.18	0.51	0.53	0.36	0.59	0.52	0.59	0.45	0.65	0.42	0.37	1.05

[a] Correlations and regressions were calculated from data obtained from individual animals for each measure of response to total iron intake. For example, the equation $Y = 1.66X + 4.03$ expresses the relationship between final hemoglobin concentration (Y) and dietary iron intake (X) for the 18 animals given ferrous sulfate as a source of iron for a 3-day regeneration period. [b] Number of days on regeneration diets.

precision in the dose–response relationship as was obtained with the standard diets.

The metameters of hemoglobin generally gave higher dose–response correlations than did the measures of packed cell volume and red blood cell count. This undoubtedly was due in part to the accuracy with which the different hematological parameters can be measured. However, biological control of hemoglobin synthesis is not identical with that of red blood cell production and variability among animals in response of the two systems to dietary treatment may have accounted for some of the difference.

Among the three estimates of hemoglobin response shown in Table III, correlation with iron intake was improved with each mathematical adjustment for individual animal differences. Change in hemoglobin concentration during the regeneration period, as opposed to final hemoglobin concentration, allowed for differences between animals in initial hemoglobin content. This resulted in increased correlation between dose and response for the rats fed diets containing bread particularly. Net gain in total hemoglobin takes into account not only differences in initial hemoglobin content but also differences in weight gain, and thus in expansion of blood volume during the repletion period. Although calculation of net hemoglobin gain involves four measurements, each of which is subject to error, the correction for variation among animals was sufficient to improve the correlation between dose and response. In this experiment, then, the highest dose–response correlations were obtained for net hemoglobin gained by animals fed the regeneration diets for 7 or 11 days.

Table IV. Ratio of Hemoglobin Iron Gained to Iron Intake by Anemic Rats

	mg of Hb-Fe gained/mg of Fe intake		
Fe source	3[a]	7	11
Ferrous sulfate	0.614	0.641	0.677
Bread	0.166	0.307	0.357
RBV	0.27	0.48	0.53

[a] Number of days on regeneration diets.

Estimates of the biological value of iron in bread relative to that of iron in ferrous sulfate are shown in the bottom line of Table III. There are no substantial differences between the estimates of RBV obtained for the three measures of hemoglobin regeneration after 7 or 11 days feeding of the repletion diets. Thus, though the precision of the estimate of RBV was improved by the mathematical adjustments for individual animal variation, there was little change in the accuracy of its estimation. Evaluations of RVB of bread iron from data on packed cell volume and red blood cell count are more variable, probably because of the poor correlation between dose and response. A 3-day regeneration period was not adequate in this study for reliable estimates of the RBV of bread iron.

Several authors (Ranhotra et al., 1971; Anderson et al., 1972; Mahoney et al., 1974) have suggested a ratio of hemoglobin (or hemoglobin iron) gain per unit of iron intake as a measure of bioavailability. This ratio has decided advantages when a variety of iron sources are being tested at one dose level. In many food items that are of nutritional interest iron content is 10 mg or less per 1000 kcal so that iron intake by the experimental animal would

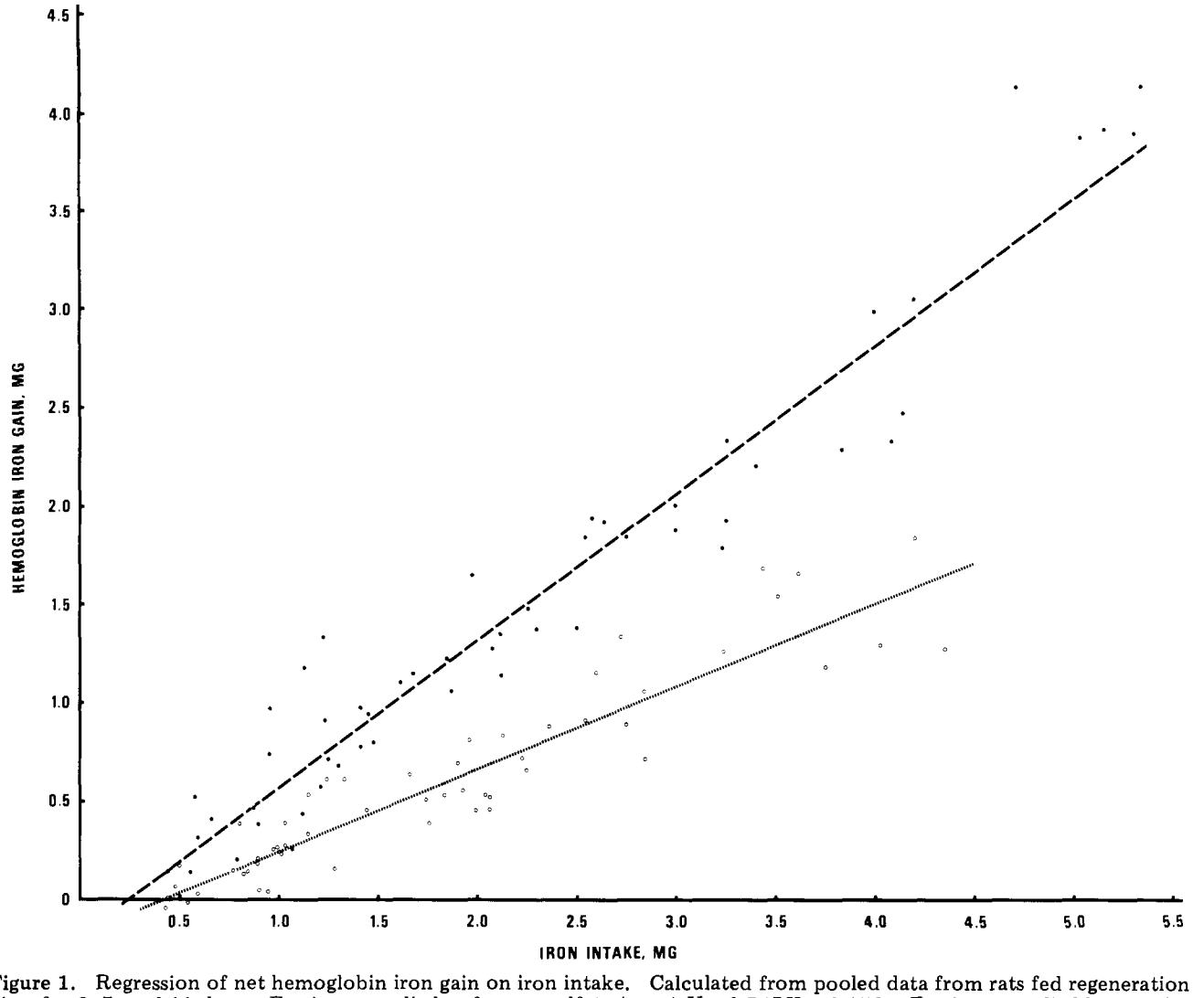

Figure 1. Regression of net hemoglobin iron gain on iron intake. Calculated from pooled data from rats fed regeneration diets for 3, 7, and 11 days. For iron supplied as ferrous sulfate (•- - -) $Y = 0.747X - 0.172$. For iron supplied by bread (○· · ·) $Y = 0.421X - 0.168$.

be limited by the caloric density of the diet. In such circumstances, expansion of the blood volume by growth of the animal would account for a major portion of the iron absorbed. Net hemoglobin iron gained per milligram of iron intake was calculated for the two iron sources and three regeneration periods used in this study (Table IV). These data again show that animals fed the bread diets were not able to absorb enough iron in 3 days to achieve the ultimate rate of hemoglobin synthesis that would later be attained on these diets. Approximately two-thirds of the iron consumed by rats fed the diets supplemented with ferrous sulfate was utilized for hemoglobin synthesis. In animals given bread as a source of iron for 7 or 11 days, about one-third of the iron intake was converted to hemoglobin, thus again indicating a RBV of about 50% for bread iron.

The simple ratio of response to dose, e.g. net hemoglobin iron gained per unit of iron intake, is a special form of regression in which it is assumed that the regression line passes through the origin of the axes. The assumption is not valid in this case (Figure 1). Since the true equation intercepts the ordinate at a point below zero, values of the ratio will be low for small gains in hemoglobin iron and will approach the value of the regression coefficient as hemoglobin iron gain increases. It is quite likely that the true relationship of hemoglobin regeneration to iron intake is sigmoidal in shape. The initial lag in the dose–response curve could correspond to the time required for maturation of erythroblasts. Although circulating reticulocytes in anemic animals might synthesize some hemoglobin, the maximum rate of regeneration would not be observed until erythrocytes formed after the increase in iron absorption began were released into the peripheral blood. It is also possible that the lag could have resulted in part from a deficiency of some iron-dependent enzymes in the tissues of these severely anemic animals. When more dietary iron first became available, the enzyme deficit would thus have been corrected at the expense of hemoglobin synthesis.

In assessing the bioavailability of iron by hemoglobin regeneration in anemic rats both the dose levels and regeneration time need to be selected so that the results fall within the linear portion of the dose–response curve. This study indicates that for animals with initial hemoglobin levels of about 5% total iron intake should be in the range of 1.5 to 4 mg. With iron concentrations of about 10 mg/1000 kcal, foods such as bread can be incorporated into animal diets at rates to achieve this intake in 11 days, and possibly less. In order for an estimate of RBV to be accepted with confidence, each different type of food would have to be fed at several dose levels and the data subjected to regression analysis. However, for more routine testing of availability of iron from a variety of similar food preparations (e.g., wheat breads) one dosage level for each iron source might suffice. In this case the total iron intake

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.

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

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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

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