way of determining how far the smaller particles advance into the virus region was done by centrifuging an essentially virus-free preparation and determining the CF activity in the lower region of the tube. Such a sample was obtained as follows: The supernatant fluid from a moving boundary ultracentrifugation, which pelleted most of the virus, was subjected to zone centrifugation to separate out only the smaller-particle fraction. The results of three experiments, shown in Fig. 4C, were almost identical to Fig. 4B, showing that the centrifugation which was designed to move the smaller particle through 0.3 ml. did not cause any more broadening of the initial zone than that which occurred by standing and subsequent handling. However, 0, 3 and 4% of the initial CF activity was found below the upper 2-ml. fraction. The over-all average of these three experiments and the four used for Fig. 4B shows 2% (range 0–6%) cross-contamination of the smaller CF particle into the virus fraction.

In experiments to demonstrate the amount of virus CF activity contaminating the smaller particle fraction, zone centrifugation of virus, free from the smaller particle, was followed by CF assay. This should indicate how much CF activity fails to leave the smaller-particle fraction. It was found to be difficult to obtain such a preparation, for the virus breaks down into smaller particles with handling.[8,20–22] Since only small percentages of cross-contamination are to be measured, virus degradation during the dialysis necessary to remove sucrose was great enough to preclude establishing contamination limits.

Discussion

These experiments show that the major 14 *S* and 140 *S* particles can be separated in one centrifugation but do not establish that there are only two particle types in the FMDV system. The presence of other types might be ascertained by recycling the intermediate fractions from the first zone ultracentrifugation. Persistence of the activity at some level other than that corresponding to the 14 *S* and 140 *S* zones would indicate such a component. Verification could be obtained by measurement of its *s*-rate by the moving boundary technique as illustrated in Fig. 1.

A distinction must be made between the percentage of a component that contaminates another fraction and the percentage of an observed activity that is due to a contaminant. Thus, if 5% of the smaller particle is in the virus region and the virus represents only 5% of the original activity, then half of the activity in the virus fraction is due to the smaller particle. When zone centrifugation for the preparation of antigens free from each other is applied, an additional zone centrifugation of each major fraction from the first cycle presumably will reduce the contamination even further.

In explanation of the differences between the *s*-rate found here for the smaller CF particle and that reported by Bradish, *et al.*,[6] the limit of error of both results should be considered. Bradish, *et al.*,[6] derived the expression for the error in the *s*-rate due to an error in the assay, when using the two-compartment formula. Their expression is slightly different from that which follows from the dr^* of equation 5 but indicates that the accuracy depends upon the upper compartment's being very nearly emptied. In their five experiments the ratio of c/c^0 was between 0.39 and 0.63, with an average of 0.52. The corresponding averages of r_a and r were 71.2 and 87.8 mm., respectively. These data give an error, ds/s, of $\pm 27\%$ using an assumed error of 25% in the assay (their $n = 1.25$). Thus, their reported value of 7.8 *S* can be no better than ± 2.1 *S* by their own argument; however this does not completely resolve the difference. There is, of course, the possibility that there is a real difference between virus strains, since their work was on type O, or that they were dealing with a breakdown product of the smaller particle itself.

GREENPORT, NEW YORK

(20) J. B. Brooksby, in the second symposium of the Society for General Microbiology (1952) "The Nature of Virus Multiplication," Cambridge Univ. Press, 1953, p. 246.

(21) F. Brown and J. Crick, *Virology*, **5**, 133 (1958).

(22) J. B. Brooksby, "Advances in Virus Research," Vol. V, K. M. Smith and M. A. Lauffer, ed., Academic Press, Inc., New York, N. Y., 1958, p. 34.

[CONTRIBUTION FROM THE BIOLOGICAL LABORATORIES, HARVARD UNIVERSITY]

The Association of Divalent Cations with Glutathione[1]

BY R. BRUCE MARTIN AND JOHN T. EDSALL

RECEIVED FEBRUARY 19, 1959

Glutathione may combine with metallic ions, at two loci: either like an α-amino acid, by chelation through the amino and carboxylate groups of the glutamyl residue, or through binding at the cysteinyl sulfur. The interactions of several divalent metallic ions with glutathione and S-methylglutathione have been determined by titration. Since the sulfur atom is blocked in the S-methyl derivative, comparison of the data permits estimation of the relative importance of binding at the two loci in glutathione. For divalent Zn, Ni, Co and Mn it is estimated that 80, 10, 30 and 15%, respectively, of the bound metallic ion is attached at the sulfur atom.

In this paper we are concerned with the binding of certain divalent metal ions to glutathione; they may be attached either to the amino and carboxyl groups of the glutamyl residue or to the sulfhydryl group of the cysteinyl residue. We have studied binding by titration with base, with and without added metal ions. To formulate the relations involved, we consider first the acid–base equilibria involving the amino and sulfhydryl groups

(1) This work was supported by grants from the United States Public Health Service (H-3169) and the National Science Foundation (G-3230).

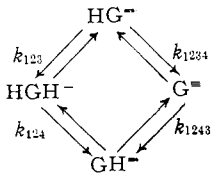

Fig. 1.—Scheme for the basic ionization of the sulfhydryl and amino groups in glutathione.

of glutathione. The third and fourth "macroscopic" ionization constants of glutathione (pK_3 and pK_4) involve the ionization of these groups. The resolution of these two constants into four "microconstants" allows an estimate to be made of the relative affinity for hydrogen ion of each microscopic form.[2]

In Fig. 1 the symbol HGH^- represents a glutathione molecule with both carboxyl groups ionized and the sulfhydryl and ammonium groups in their acidic form. The symbol $HG^=$ represents a glutathione molecule with the sulfhydryl group, in addition to the carboxyl groups, ionized, the ammonium group being still positively charged. The symbol $GH^=$ represents the microscopic form with only the -SH group un-ionized, whereas the symbol $G^{\equiv}$ represents a molecule of completely ionized glutathione. The microscopic ionization constants are defined as, $k_{123} = (H^+)(HG^=)/(HGH^-)$, etc. The explanation of the particular subscripts used for the microconstants, their relation to the macroconstants obtained by titration and the evaluation of the microconstants by the use of the model compound S-methylglutathione has previously been described.[2] The values obtained at 25° and 0.16 μ for the constants in Fig. 1 are: $pk_{123} = 8.92$, $pk_{124} = 9.20$, $pk_{1243} = 9.16$ and $pk_{1234} = 9.44$.

In this report the S-methyl derivative of glutathione is used to obtain the association constants of divalent cations with the amino and carboxyl groups of the γ-glutamyl residue. These constants may then be taken as known for the study of the interaction of the metal ions with glutathione itself. Hence the binding to the sulfhydryl group may be separated from that to the γ-glutamyl moiety. Chelation between these groups is not considered likely because of the large ring involved. No data were obtained which necessitated the postulation of the ionization of a peptide hydrogen. However, chelation at a peptide bond is possible with binding at the sulfur atom.

Cupric ions oxidize glutathione and were not studied. Cadmium forms complexes with glutathione too insoluble for measurement by potentiometric methods, but it is probable from studies on other compounds that the binding at the sulfur atom is predominant over that at the γ-glutamyl locus. Magnesium is bound only weakly and was not considered further. This report concerns mainly the divalent ions, zinc, nickel, cobalt and manganese.

(2) R. B. Martin and J. T. Edsall, *Bull. soc. chim. biol.*, **40**, 1763 (1958). For a discussion of the general meaning and determination of the macro- and microconstants, see J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. 1, Academic Press, Inc., New York, N. Y., 1958, Chapter 9.

Experimental

The glutathione, the preparation of S-methylglutathione, the reagents and instruments employed, have previously been described.[2] The metal ions were added as their nitrates. Standard base was added from a Gilmont ultramicroburet to a mixture containing the desired quantities of ligand, metal nitrate and potassium nitrate to yield an ionic strength of 0.16. The *p*H was recorded after additions of standard base. The temperature was 25 ± 1°.

Results

Many data were recorded, and only a few typical experiments are reported here. Figures 2 and 3 show the effect on the titration curve of adding one-

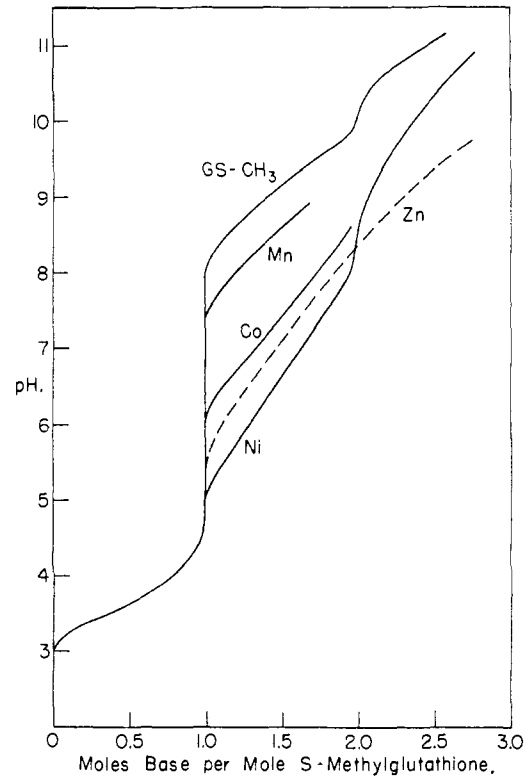

Fig. 2.—Titration of S-methylglutathione with divalent cations at 25°; 3.0 ml. of solution 0.01 *M* in G-S-CH_3, 0.005 *M* in metal nitrate (absent in upper curve), 0.14 *M* in KNO_3, titrated with 0.1510 *M* NaOH. Cobaltous and manganous ions yielded precipitates at the points of termination of the curves.

half mole of the indicated divalent cation per mole of S-methylglutathione and glutathione, respectively. The identity of the titration curves with and without added metal ion for the first equivalent of base added, either to glutathione or its S-methyl derivative, indicates that neither carboxyl group functions by itself as a binding locus. Only upon the addition of the second and third equivalents of base per mole of glutathione is the titration curve displaced to more acid *p*H values on the addition of metal ion.

The titration curves obtained with S-methylglutathione were analyzed by standard methods[3] to yield the formation constants, k_{N1}, listed in Table I. Only the binding with the $GH^=$ form of Fig. 1 need be considered in this case. Mixtures

(3) J. T. Edsall, G. Felsenfeld, D. S. Goodman and F. R. N. Gurd, THIS JOURNAL, **76**, 3054 (1954).

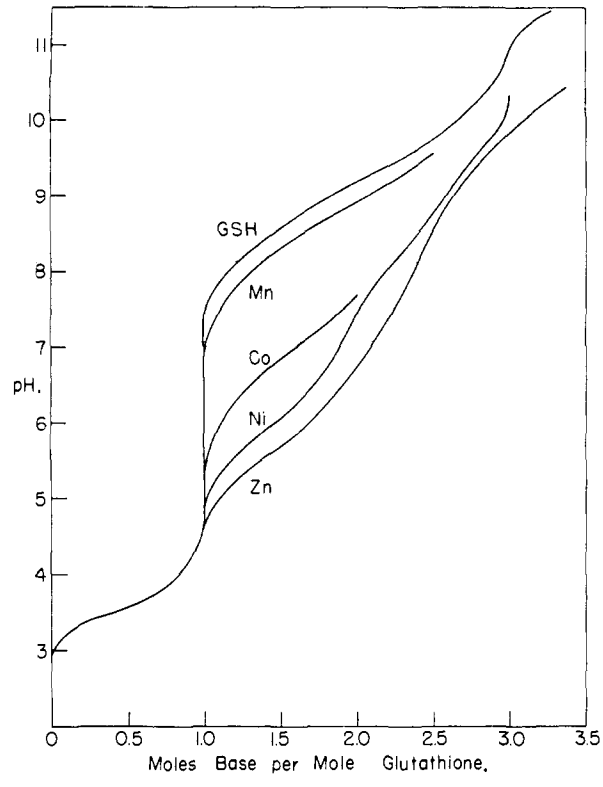

Fig. 3.—Titration of glutathione with divalent cations at 25°; 3.0 ml. of solution 0.02 M in GSH, 0.01 M in metal nitrate (absent in upper curve), 0.14 M in KNO_3, titrated with 0.1510 M NaOH. The cobaltous ion curve was not reproducible after about pH 7.5 and manganous ion yielded a precipitate at the termination of the curve.

with varying ratios of ligand to metal were titrated and all gave identical results within the experimental error. No attempt was made to look for complexes with a ligand to metal ratio greater than two. The values of k_{N1} listed in Table I are what might be expected for an α-amino acid of pK = 9.20. They are in general slightly lower than, but not very different from, the values listed by Gurd and Wilcox[4] for the combination of the glycinate anion with the same divalent metallic ions studied by us.

The interpretation of the data for glutathione is more complex and will be considered in some detail. Initially, the metal ions combine with glutathione in one of two ways, and in the notation of Fig. 1 these may be written as

$$M^{++} + GH^{=} \rightleftharpoons MGH \qquad k_{N1} = (MGH)/(GH^{=})(M^{++}) \quad (1)$$

$$M^{++} + HG^{=} \rightleftharpoons HGM \qquad k_{S1} = (HGM)/(HG^{=})(M^{++}) \quad (2)$$

where k_{N1} is the first formation constant for binding at the γ-glutamyl locus, as evaluated from the study with S-methylglutathione, and k_{S1} is the first formation constant for binding at the sulfur atom.

A second mole of ligand may combine with the complex MGH also at the γ-glutamyl locus as

(4) F. R. N. Gurd and P. E. Wilcox, *Advances in Protein Chem.*, **11**, 311 (1956). See their Table III, p. 329.

$$MGH + GH^{=} \rightleftharpoons GHMGH^{=} \qquad k_{N2} = (N2^{=})/(GH^{=})(MGH) \quad (3)$$

where $(N2^{=})$ is a shorthand notation for the molar concentration of the complex of two ligands both bound through the γ-glutamyl locus and k_{N2} is assumed to be equal to the second formation constant as obtained from the S-methyl derivative.

Similarly, the complex HGM may add a second mole of ligand at the sulfur atom as

$$HGM + HG^{=} \rightleftharpoons HGMHG^{=} \qquad k_{S2} = (S2^{=})/(HG^{=})(HGM) \quad (4)$$

A mixed complex may also form as

$$HGM + GH^{=} \rightleftharpoons HGMGH^{=} \rightleftharpoons HG^{=} + MGH$$

Representing the molar concentration of the mixed complex in the center as $(SN^{=})$, two more equilibrium constants may be defined as k_{SN} and k_{NS}

$$k_{SN} = (SN^{=})/(HGM)(GH^{=}) \quad (5)$$

$$k_{NS} = (SN^{=})/(MGH)(HG^{=}) \quad (6)$$

from which it follows that $k_{NS}/k_{SN} = k_{S1}/k_{N1}$.

The total molar concentration of metal in the system is

$$C_M = (M^{++}) + (HGM) + (MGH) + (N2^{=}) + (S2^{=}) + (SN^{=}) \quad (7)$$

The total molar concentration of ligand is

$$C_G = (HGH^{-}) + (HG^{=}) + (GH^{=}) + (G^{\equiv}) + (HGM) + (MGH) + 2(S2^{=}) + 2(N2^{=}) + 2(SN^{=}) \quad (8)$$

Combination of equations 7 and 8 with the electroneutrality relationship yields

$$(HGH^{-}) = 2C_G + (OH^{-}) - (Na^{+}) - (H^{+}) + (G^{\equiv}) \quad (9)$$

where (Na^{+}) is due to the added NaOH. For the results reported here the $(G^{\equiv})$ term is negligible.

Define a total $\bar{\nu}$ as the average number of moles of γ-glutamyl and sulfur groups bound per mole of metal present in all forms

$$\bar{\nu}C_m = (HGM) + (MGH) + 2(S2^{=}) + 2(N2^{=}) + 2(SN^{=}) \quad (10)$$

Substitution of the appropriate equilibrium expressions, making use of equations 1–10 yields a function denoted as Q_2

$$Q_2 = \frac{\bar{\nu}}{(2-\bar{\nu})(HG^{=})} = \frac{(k_{S1}+0.53k_{N1})+2[k_{S1}k_{S2}+0.53k_{N1}k_{NS}+(0.53)^2k_{N1}k_{N2}](HG^{=})}{2+(k_{S1}+0.53k_{N1})(HG^{=})} \quad (11)$$

where the factor 0.53 arises from the replacement of $(GH^{=})$ with $0.53(HG^{=})$. At low pH values, and hence at low values of $\bar{\nu}$, the value of $(HG^{=})$ approaches zero, and Q_2 under these conditions approaches a constant which we denote as Q_1.

$$Q_1 = k_{S1} + 0.53k_{N1} \quad (12)$$

Thus

$$Q_2 = \frac{Q_1 + 2B(HG^{=})}{2 + Q_1(HG^{=})} \quad (13)$$

where B is the expression in brackets in the equation for Q_2 in (11) above. Therefore a plot of log Q_2 *versus* $\bar{\nu}$ extrapolates to log $(Q_1/2)$ at $\bar{\nu} = 0$.

For the equimolar mixtures of metal ion and ligand log $Q_1 = \log[\bar{\nu}/(1-\bar{\nu})(HG^{=})]$ was also extrapolated to $\bar{\nu} = 0$, since the values of $\bar{\nu}$ approaching unity are affected by subsequent ionizations of the com-

plex and by the possibility, not considered here, that one glutathione molecule may bind two metal ions.

The values obtained for log Q_1 were identical, within experimental error, for mixtures with a ligand to metal ratio from 1:1 to 8:1. The value given in Table I for log k_{N1} and log Q_1 are stated to the nearest 0.05 log unit and the estimated accuracy is $\pm$ 0.1 in the logarithm. A greater accuracy cannot be assumed, due to the limits of purity of the glutathione used and due to the cumulation of errors in those constants dependent upon others previously obtained. The values of log k_{S1} were calculated from the value of k_{N1} obtained from the data on S-methylglutathione and use of equation 12. These values are subject to large error, due to their small value, in all the complexes with metals other than zinc. In the last column of Table I an estimate is made of the percentage of the metal binding at the sulfur atom as compared with the γ-glutamyl locus.

TABLE I

LOGARITHM OF FORMATION CONSTANTS FOR DIVALENT CATIONS WITH S-METHYLGLUTATHIONE AND GLUTATHIONE AT 25° AND 0.16 μ

Metal	log k_{N1}	(log Q_1)$_{\bar{r}=0}$	log k_{S1}	% Metal at S
Zn	4.65	5.10	5.00	80
Ni	5.25	5.00	4.0	10
Co	4.30	4.20	3.7	30
Mn	2.85	2.65	1.9	15

In the case of a 2:1 mixture of glutathione and zinc, where the binding at the sulfur is predominant, an approximate pK value for the first ionization constant of a proton from the complex is 7.5; this value is inferred from the segment of the titration curve for Zn in Fig. 3 between 2.0 and 2.5 equivalents of added base. This pK value may be compared with the value of 7.85 obtained for the same ionization from the Hg(II)–glutathione$_2$ complex,[5] where the binding is almost exclusively at the sulfur.

(5) W. Stricks and I. M. Kolthoff, THIS JOURNAL, **75**, 5673 (1953).

The evaluation of the term in brackets in the expression for Q_2 was not considered worth while because of overlap with the ionizations of the complexes formed, the possible formation of higher complexes and the possibility of oxidation at the higher pH values. The ionization of protons from the complexes formed in equimolar mixtures is complicated by hydroxy complex formation and precipitation in some cases.

Discussion

The interpretation of the data in the pH region below 8 is fairly certain, but this is not so for the less reproducible values in the higher pH regions, which have not been considered in this report. The data at lower pH have provided valuable information on the distribution of metal ion between the alternative γ-glutamyl and thiol binding sites in glutathione. This behavior is probably similar to that of the higher complexes even though some of them are of the mixed type.

The relatively greater affinity of the sulfur atom of zinc as compared with nickel is similar to what is found in other sulfur compounds with no amino chelation.

The significance of the structure of glutathione for its biological function often has been discussed. The great effectiveness of the γ-glutamyl locus in competing with the sulfur atom for metal ions suggests that the γ-glutamyl residue may function as a relatively strong point of attachment for certain metal ions, thus permitting the sulfur atom to remain relatively free and hence active. This arrangement, which is essentially equivalent to placing an α-amino acid permanently close to the cysteine–SH group, performs this "protective" function far better than would be possible with an ordinary cysteinyl peptide containing only –CO-NH– linkages arising from α-carboxyl and α-amino groups. Whether this is indeed a significant aspect of the biological function of glutathione is a matter for further inquiry.

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[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF PITTSBURGH SCHOOL OF MEDICINE]

Some Consequences of the "Non-competitive" Inhibition by Glucose of Rat Liver Glucose 6-Phosphatase[1]

BY HAROLD L. SEGAL

RECEIVED OCTOBER 7, 1958

A mechanism for glucose 6-phosphatase action is presented which accounts for both the enzyme-catalyzed exchange of C^{14}-glucose with glucose 6-phosphate and the "non-competitive" type of inhibition by glucose. Several other reaction pathways are considered which would also account for the exchange reaction and shown not to be applicable on the basis of the kinetic data or to be unlikely from other considerations. The dissociation constant of the enzyme–substrate complex and standard free energy change for substrate binding to the enzyme are determined, as well as other kinetic parameters of the system.

We have reported[2] recently that glucose produces an inhibition of the glucose 6-phosphatase reaction of rat liver of a type which is traditionally referred to as "non-competitive"; that is, the presence of the inhibitor decreases the apparent maximum velocity at "infinite" substrate concentration (V_m') but does not alter the substrate concentration for half-maximum velocity (K_s'). One mechanism which leads to kinetics of this type is that in which the inhibitor combines with the

(1) This investigation was supported by a research grant (A-875) from the National Institutes of Health, U. S. Public Health Service.

(2) H. L. Segal, M. E. Washko and C. W. Lee, *Science*, **128**, 251 (1958).