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

BY HAROLD L. SEGAL

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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).

enzyme with an affinity which is independent of the presence of the substrate on the enzyme.[3] A corollary of this relationship is that the presence of the *inhibitor* on the enzyme has no effect on the binding of the *substrate* to the enzyme, although it completely prevents its breakdown into products. Such a combination of events seems self-contradictory, and in fact unambiguous examples of this mechanism are difficult to find. Laidler[4] has listed several cases of "non-competitive *p*H dependence," but this is obviously a special case. On the other hand, in reactions involving an activator or second substrate, an inhibitor may be non-competitive with one of the components but fully competitive with the other (see for example, Bernfeld, *et al.*[5]).

In the present case, we considered the possibility that glucose was non-competitive with glucose 6-phosphate (G-6-P) but competitive with the second substrate, H_2O, as postulated for a number of analogous cases of hydrolases with group transfer activity.[5a] That is, the phosphoryl group could be transferred to a hydroxyl ion or another molecule of glucose. In the latter case there would be an incorporation of C^{14}-labeled glucose into hexose phosphate. Hass and Byrne[6] have found this exchange to occur and we have now confirmed their observation. In all cases, the amount of C^{14}-glucose incorporated was equivalent to the difference in the amount of phosphate released as orthophosphate (P_i) in the uninhibited and inhibited reactions (Table I) as predicted from the mechanism postulated below.

TABLE I

EXCHANGE OF C^{14}-GLUCOSE WITH G-6-P

Each incubation tube contained in 2.0 ml., 0.6 ml. of 0.25 *M* cacodylate buffer, *p*H 6.4, 0.4 ml. of a soluble glucose 6-phosphatase preparation from rat liver[7] containing 0.93 mg. of protein, plus additions noted. G-6-P was added as the potassium salt. The C^{14}-glucose contained 14,840 c.p.m./μmole. Incubation was for 1 hr. at 30°. The reaction was stopped by the addition of 0.4 ml. of 30% trichloroacetic acid (TCA). After centrifugation, an aliquot of the supernatant fluid was analyzed for P_i by the method of Fiske and SubbaRow.[8] G-6-P was isolated from another aliquot by the method of LePage,[9] counted in a gas flow counter and analyzed for G-6-P by the difference between total and inorganic phosphate.

Additions	P_i formed (μmoles)	P_i inhib. (μmoles)	C^{14}-gluc. incorp. (c.p.m./ μmole G-6-P isol.)	Total C^{14}-gluc. incorp. into G-6-P (μmoles)
20 μmoles G-6-P	2.17	0.00	..	...
20 μmoles G-6-P + 62 μmoles C^{14}-glucose	1.74	0.43	298	0.41[a]
3.3 μmoles P_i + 62 μmoles C^{14}-glucose	..	..	11[b]	0.01

[a] Calcd. on basis of G-6-P added since extent of hydrolysis was small. [b] 10 μmoles of carrier G-6-P was added to 1.0 ml. of the TCA sup. fluid prior to isolation.

SCHEME I

$$E + G\text{-}6\text{-}P \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\cdot G\text{-}6\text{-}P$$

$$E\cdot G\text{-}6\text{-}P \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\cdot P + \text{glucose}$$

$$E\cdot P + OH^- \xrightarrow{k_5} E + P_i$$

The steady-state rate equation for this reaction sequence is

$$v_0' = \frac{\dfrac{k_3k_5(E_0)}{k_4(Glu) + k_5 + k_3}(G\text{-}6\text{-}P)}{\dfrac{k_2k_4(Glu)/k_1 + k_5(k_2 + k_3)/k_1}{k_4(Glu) + k_5 + k_3} + (G\text{-}6\text{-}P)} \quad (1)$$

where

v_0' = initial velocity of the inhibited reaction
(E_0) = total enzyme concentration
(Glu) = glucose concentration

Haas, *et al.*,[10] have developed an equivalent rate equation for this reaction sequence.

The finding of "non-competitive" kinetics in this system,[2] however, poses a restriction on equation 1; namely, that the term in the denominator

$$\frac{k_2k_4(Glu)/k_1 + k_5(k_2 + k_3)/k_1}{k_4(Glu) + k_5 + k_3}$$

which is equal to K_s' must be independent of (Glu). The most plausible relationship which satisfies this requirement is that $k_3 << k_2$ and $k_3 << k_5$. Then

$$v_0' = \frac{\dfrac{k'(E_0)}{(Glu)/K_1 + 1}(G\text{-}6\text{-}P)}{k_2/k_1 + (G\text{-}6\text{-}P)} \quad (2)$$

where $K_1 = k_5/k_4$ and $k' = k_3$.

If only the first inequality exists, a partially non-competitive and partially competitive type of inhibition would occur.[11]

The implication of the first inequality is that the attainment of the equilibrium between enzyme and substrate is rapid in comparison with the rate of subsequent reactions. In this connection it may be pointed out that Morales[12] has concluded from theoretical considerations that, for the more traditional mechanism of inhibitor action also, non-competitive behavior strongly suggests the existence of an equilibrium state, in this case among *all* the various enzyme complexes.

Further experimental support for the conclusion that $k_3 << k_2$ in the present case comes from the observation that the total amount of glucose incorporated, as calculated from the difference in P_i released in the uninhibited and inhibited reactions, can be recovered quantitatively in the

(3) J. B. S. Haldane, "Enzymes," Longmans, Green and Co., London, 1930, p. 46.

(4) K. J. Laidler, *Disc. Faraday Soc.*, **20**, 83 (1955).

(5) P. Bernfeld, S. Jacobson and H. C. Bernfeld, *Arch. Biochem. Biophys.*, **69**, 198 (1957).

(5a) R. K. Morton, *Disc. Faraday Soc.*, **20**, 149 (1955).

(6) L. F. Hass and W. L. Byrne, 4ème Cong. Inter. de Biochim., Abst. No. 4–16, p. 39 (1958), and personal communication.

(7) H. L. Segal and M. E. Washko, *J. Biol. Chem.*, in press.

(8) C. H. Fiske and Y. SubbaRow, *ibid.*, **66**, 375 (1925).

(9) G. A. LePage, in "Manometric Techniques and Tissue Metabolism" (W. W. Umbreit, R. H. Burris and J. F. Stauffer, eds.), Burgess Publishing Co., Minneapolis, Minn., 1949, pp. 186–190.

(10) L. F. Hass, F. C. Neuhaus, W. L. Byrne and G. W. Schwert, personal communication.

(11) H. L. Segal, in "The Enzymes," 2nd Ed. (P. D. Boyer, H. A. Lardy and K. Myrbäck, eds.), Academic Press, New York, N. Y., 1959, pp. 33–34; H. L. Segal, J. F. Kachmar and P. D. Boyer, *Enzymologia*, **15**, 187 (1952).

(12) M. F. Morales, THIS JOURNAL, **77**, 4169 (1955).

free G-6-P at the end of the reaction. If the E·G-6-P complex did not rapidly equilibrate with free E + G-6-P in the first step of the reaction sequence, the specific activity of the complex would always be higher than that of the free glucose, and thus less than the calculated amount of C^{14}-glucose would be trapped in the G-6-P pool.

Several other conceivable reaction pathways of G-6-P hydrolysis which would account for the exchange reaction can be eliminated on the basis of the kinetic evidence.

A mechanism wherein the enzyme is phosphorylated without the intervention of a binary complex but instead directly in a second-order reaction

$$E + \text{G-6-P} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\cdot P + \text{Glu}$$

$$E\cdot P \xrightarrow{k_5} E + P_i$$

leads to the rate equation

$$v_0' = \frac{k_5(E_0)(\text{G-6-P})}{[k_5 + k_2(\text{Glu})]/k_1 + (\text{G-6-P})} \qquad (3)$$

which is the rate equation for a *competitive type* of inhibition, and the mechanism is thus ruled out in this case.

A pathway which has been suggested by Boyer and Harrison[13] for certain group transfer reactions which involves a transfer during the existence of a ternary complex of enzyme, donor and acceptor may be applied to the present case as

SCHEME II

$$E + \text{G-6-P} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\cdot\text{G-6-P}$$

$$E\cdot\text{G-6-P} + \text{glucose}^* \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \begin{cases} E\langle \text{glucose}^* \\ \quad\ \text{G-6-P} \end{cases} \quad \frac{k_3}{k_4} = K_i$$

$$k_6 \uparrow\downarrow k_5$$

$$\begin{cases} E\langle \text{G*-6-P} \\ \quad\ \text{glucose} \end{cases} \quad \frac{k_6}{k_5} = K_{eq.} = 1$$

$$E\cdot\text{G-6-P} + OH^- \xrightarrow{k_7} E + \text{glucose} + P_i$$

This mechanism implies a labilization of the phosphoryl group of G-6-P to a nucleophilic attack by either a hydroxyl ion or another molecule of glucose without the intermediate formation of a discrete enzyme–phosphate compound. Boyer and Harrison[13] have pointed out that exchange reactions of this type might be expected when there is a low order of specificity toward the group acceptor.

The rate equation for this pathway

$$v_0' = \frac{\dfrac{k_7(E_0)}{[1 + (\text{Glu})/K_i]}(\text{G-6-P})}{\dfrac{(k_2 + k_7)/k_1}{[1 + (\text{Glu})/K_i]} + (\text{G-6-P})} \qquad (4)$$

however, is of the type for *uncompetitive* inhibition, and thus the finding of non-competitive kinetics eliminates this mechanism in the present case.

(13) P. D. Boyer and W. H. Harrison, in "The Mechanism of Enzyme Action" (W. D. McElroy and B. Glass, eds.), Johns Hopkins Press, Baltimore, Md., 1954, p. 658.

A modification of this sequence in which the phosphoryl group is transferred to a second molecule of glucose in a second-order reaction without the formation of a ternary complex or an enzyme–phosphate intermediate would allow for the exchange reaction but is not consistent with an inhibition by glucose of any type.

$$E + \text{G-6-P} \rightleftharpoons E\cdot\text{G-6-P}$$

$$E\cdot\text{G-6-P} + \text{glucose}^* \rightleftharpoons E\cdot\text{G*-6-P} + \text{glucose}$$

$$E\cdot\text{G-6-P} + OH^- \longrightarrow E + \text{glucose} + P$$

On the other hand, if it is visualized in Scheme II that glucose can also combine with the enzyme *prior* to the combination of G-6-P, the finding of non-competitive kinetics would lead to a rate equation of the form of equation 2 for this mechanism, based upon the conclusion of Morales[12] that non-competitive behavior implies an equilibrium state among all the enzyme complexes. In this case, the term K_i in eq. 2 is the dissociation constant of the enzyme–inhibitor complex and k' is k_7.

This mechanism is considered less likely than that represented by Scheme I for two reasons. Firstly, the finding that the amount of glucose incorporated into G-6-P is stoichiometrically equal to the extent of inhibition of P_i appearance requires an entirely fortuitous equality in Scheme II between the rate constant for the exchange reaction (k_5) and the rate constant for the breakdown of the enzyme–G-6-P complex into products (k_7). Secondly, Scheme II requires a binding of glucose to

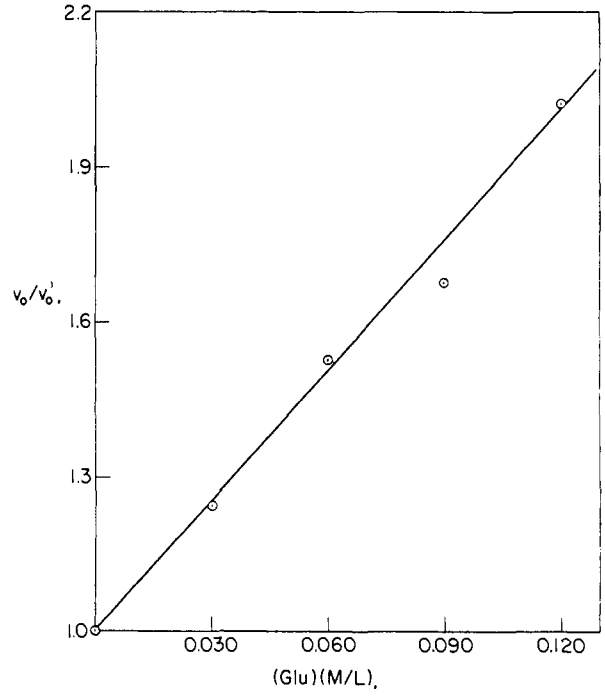

Fig. 1.—Calculation of K_i of glucose inhibition. Incubation was for 10 min. at 30° in the presence of 0.075 *M* cacodylate buffer, *p*H 6.4, 10^{-2} *M* G-6-P, 2.5 mg./ml. of homogenized rat liver, and the concentration of glucose shown. The reaction was stopped and the solution analyzed for P_i as in Table I. Slope was calculated by method of least squares.

the enzyme, in a rather specific manner, which has nothing to do with its hydrolytic function. On the other hand, the inhibition by glucose predicted from Scheme I results from its direct participation in the reaction sequence.

From equation 2 and the corresponding reaction pathway, certain kinetic and thermodynamic parameters of the system can be calculated. The average of the apparent Michaelis constants, K_s' for the solubilized enzyme system was calculated to be $0.8 \times 10^{-3} M$.[7] From equation 2, K_s' is seen to be identical with k_2/k_1, the dissociation constant of the enzyme–substrate complex. Thus, at *p*H 6.4 and 30° the standard free energy change of substrate binding is 4280 cal.

A second parameter of the system was calculated from a plot of v_0/v_0' *versus* (Glu) as shown in Fig. 1,[7] where v_0 is the velocity of the uninhibited reaction. The reciprocal of the slope of the plot, which is equal to k_5/k_4 from Scheme I, has an average value for the system from several normal rat liver homogenates of 0.12 *M*.

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[CONTRIBUTION FROM THE ORGANIC CHEMICAL RESEARCH SECTION, LEDERLE LABORATORIES DIVISION, AMERICAN CYANAMID CO.]

The Reaction of Periodate with Aminosugars. Anomalous Overoxidations of Aminofuranosides

BY MARTIN J. WEISS, JOSEPH P. JOSEPH, HENRY M. KISSMAN, ARLENE M. SMALL, ROBERT E. SCHAUB AND FRANCIS J. MCEVOY

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The reaction of sodium metaperiodate with various 3-aminoribofuranosyl derivatives and with one 3-aminoarabinofuranosyl derivative results in the consumption of two rather than the expected one molar equivalent of oxidant. Two 5-aminoribofuranosides reacted with four equivalents of periodate. Various 2- and 3-aminopyranosides reacted "normally." It is shown that the bis-aldehyde, which is obtained from the oxidation of the corresponding nonamino furanosides, is neither an intermediate nor the final product of the oxidation of the 3-aminofuranosides. The implications of this anomalous behavior for structure determinations are discussed.

Oxidative cleavage of 1,2-glycol and 1,2-aminoalcohol systems by periodate is a very convenient and useful tool for structure determinations, especially in the fields of carbohydrate and nucleoside chemistry.[1] In general, these systems react with one molar equivalent of periodate. However, we now wish to report that various 3-aminoribofuranosyl derivatives and one 3-aminoarabinofuranoside react with *two* molar equivalents of this oxidant. On the other hand, various 2- and 3-aminopentopyranosyl and hexopyranosyl derivatives have been found to react in the "normal" sense—that is, with the theoretically-required two equivalents of periodate.[2]

Overoxidation of aminofuranosides was first observed in this Laboratory with certain aminosugar derivatives, the furanoside configuration of which was likely but was not rigorously established at the time. Therefore, methyl 3-amino-3-deoxy-β-D-ribofuranoside (III) was prepared as a model aminofuranoside of unequivocal structure. This compound previously has been reported,[3] without physical data, as a low melting solid. For this study, it was obtained as a crystalline solid by a two-step synthesis in 43% over-all yield from 2,5-di-*O*-benzoyl-3-phthalimido-3-deoxy-β-D-ribofuranosyl chloride (I),[3] which is available from the antibiotic puromycin *via* the corresponding 1-*O*-acetyl derivative V. Methanol treatment of I gave a 65% yield of the blocked methyl glycoside (II), which on deblocking with methanolic butylamine[4] afforded a 67% yield of the desired methyl 3-aminoribofuranoside (III), m.p. 107–109°, $[\alpha]^{25}D$ −37° (1.1% H_2O). The assigned furanoside structure was unequivocally verified by N-phthaloylation in 58% yield to the previously reported[3] methyl 3-phthalimido-3-deoxy-β-D-ribofuranoside (IV) and also by conversion of IV to 1-*O*-acetyl-2,5-di-*O*-benzoyl-3-phthalimido-3-deoxy-β-D-ribofuranoside (V)[3] by benzoylation followed by acetolysis with acetic acid–acetic anhydride–sulfuric acid. This last sequence (III → IV → V) previously has been carried out starting with crude III.[3]

Periodate treatment of methyl 3-aminoribofuranoside (III) confirmed our original impression that 3-aminofuranosides would consume two, rather than the anticipated one, molar equivalents of oxidant. Thus, reaction of III with sodium metaperiodate in aqueous solution at room temperature resulted in an uptake of two molar equivalents of oxi-

(1) See E. L. Jackson, ["Organic Reactions," Vol. II, 1944, p. 341] for a general review of periodic acid oxidation. This subject is also reviewed with especial reference to its application in carbohydrate chemistry by J. M. Bobbitt, [*Advances in Carbohydrate Chemistry*, **11**, 1 (1956)] and J. R. Dyer ["Methods of Biochemical Analysis," Vol. III, 1956, p. 111]. The use of periodate oxidation for the determination of nucleoside anomeric configuration was first described by J. Davoll, B. Lythgoe and A. R. Todd, [*J. Chem. Soc.*, 833 (1946)].

(2) G. E. McCasland and D. A. Smith [THIS JOURNAL, **73**, 5164 (1951)] studied the reaction of the *cis*- and *trans*-2-aminocyclohexanols and 2-aminocyclopentanols with lead tetraacetate and also with sodium periodate. They observed over-oxidation of the aminohexanols and of the aminopentanols on treatment with lead tetraacetate. Their periodate studies were carried out with equivalent amounts of oxidant and aminoalcohol and, therefore, over-oxidation could not be observed. The periodate oxidation of inosamines proceeds as expected with the uptake of 6 molar equivalents of oxidant [T. Posternak, *Helv. Chim. Acta*, **33**, 1597 (1950); H. Straube-Rieke, H. A. Lardy and L. Anderson, THIS JOURNAL, **75**, 694 (1953); G. R. Allen, Jr., of this Laboratory, unpublished results]. The only reported exception is D,L-*epi*inosamine-2 which consumes approximately 8 molar equivalents after 50 hours (H. Straube-Rieke, *et al.*, above).

(3) B. R. Baker, J. P. Joseph and R. E. Schaub, *ibid.*, **77**, 5905 (1955).

(4) L. Goldman, J. W. Marsico and R. B. Angier, *ibid.*, **78**, 4173 (1956).