with the nearly quantitative recovery of the unaltered glycoside from urine and feces.

Summary. Urinary and fecal excretion of the naturally occurring toxic and carcinogenic glycoside, cycasin, β-D-glycosyloxyazoxymethane, was determined in germfree and conventional Sprague-Dawley male rats. Excretion in germfree rats was nearly quantitative while only 18 to 35% was recovered in conventional rats. Since cycasin is inert until acted upon by a β-glucosidase, the lack of enzymatic cleavage in germfree rats suggests the bacterial flora as the most likely source for the enzyme in conventional rats. Considerable variation was encountered in the amount of cycasin which was not recovered and was presumably metabolized in conventional rats. The possible significance of this variability is discussed in relation to toxicity and carcinogenicity.

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Formation Constants of Certain Zinc-Complexes by Ion-Exchange Method.* (30794)

PRAN VOHRA, ELLEN KRANTZ AND F. H. KRATZER
Department of Poultry Husbandry, University of California, Davis

The effectiveness of disodium ethylenediaminetetraacetic acid (EDTA) in improving the availability of zinc has been shown for poults(1) and chicks(2,3) fed diets containing isolated soybean protein. The optimum stability constant for a chelating agent to be effective was found to be between 13 and 17, using maximum values from the literature (4). To explain the mechanism of the biological action of chelating agents it is desirable to know the formation constants for additional compounds and to have these values for a physiological pH.

Let us consider a general reaction between zinc ion and any complexing agent *A* with a valency of x which can be described by the following equation:

$$Zn^{++} + nA^{-x} = Zn(A)_n^{2-xn} \qquad (1)$$

The formation constant for this reaction is:

$$K_{f\ (absolute)} = \frac{[Zn\ (A)_n^{2-xn}]}{[Zn^{++}]\ [A^{-x}]^n} \qquad (2)$$

The log value of K_f is called the stability or the formation constant for the reaction. The values for formation constants are usually determined under well-defined conditions of ionic strength, optimal pH at which the complexing agent is present in its completely dissociated form A^{-x}, and a temperature of 20°C. Such conditions are seldom attainable in studies involving biological systems in intact animals.

As the biologists are more interested in the relative value of the formation constants at about the physiological pH of 7.4, the current investigation was undertaken following the procedures outlined by Schubert(5). The values so determined are termed as apparent formation constants and are related to the absolute formation constants as follows:

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$$K_{f\ (apparent)} = \frac{[Zn\ (A)_n^{2-xn}]}{[Zn^{++}]\,[A^{-x}]^n a_H a_A} \quad (3)$$

$$= K_{f\ (absolute)}/a_H a_A \quad (4)$$

where a_H compensates for the presence of other protonated forms of A and a_A compensates for the presence of other complex forming substances besides the one under investigation.

Experimental. Materials. Carrier free $Zn^{65}Cl$ was obtained from Oak Ridge National Laboratory. Hydroxyethylethylenediaminetetracetic acid (HEDTA), ethylenediaminetetraacetic acid (EDTA), Terephthalic acid (TP), citric acid, nitrilotriacetic acid (NTA), and glycolic acid were products of Eastman Organic Chemicals. Sodium acid pyrophosphate, sodium hexametaphosphate and sodium tripolyphosphate were purchased from Alfa Inorganics, Inc., Beverly, Mass. Ethylenediaminediacetic acid (EDDA), 1,2 diaminocyclohexanetetraacetic acid (CDTA), ethylenediaminebitartrate (EDBT) were purchased from K & K Labs., Inc., Jamaica, N. Y. Diethylenetriaminepentaacetic acid (DTPA), dihydroxyethylenediaminediacetic acid (DHEEDA) and thiopropionic acid (TPA) were donated by Dow Chemical Co., Midland, Mich., Dr. A. Wallace, U.C.L.A., Los Angeles, and American Cyanamid Co., New York, respectively. 2-Hydroxypropylenediaminetetraacetic acid (HPDTA) was bought from Aldrich Chemical Co., Milwaukee, Wis., and ethylenediamine -N, N'-diacetic acid -N, N'-dipropionic acid (EDDADP) was prepared in the laboratory(4).

Procedure. The apparent formation constants (to be referred as K_f) were determined by the ion-exchange method of Schubert(5) using Dowex-50 cation-exchanger equilibrated with 0.16 M NaCl solution. Carrier-free $Zn^{65}Cl$ was added to the 5,5-diethylbarbituric acid buffer of pH 7.4 to give approximately 10,000 C.P.M./ml. The equilibration experiments were carried out in duplicate in 125 ml Erlenmeyer flasks. Each flask contained 200 mg ion exchange resin (sodium form), 10 ml of buffer solution containing Zn^{65}, a known concentration of the chelating agent diluted in 0.16 M NaCl and the necessary amount of 0.16 M NaCl to make the volume 50 ml. The equilibration was carried out by stirring the flasks for 3 hours at room temperature (about 25°C) on a rotary wheel mounted at 45° angle. After settling the resin, 0.5 ml aliquots of supernatant solution were pipetted into vials containing 15 ml scintillating fluid and were counted in a 2 channel liquid scintillation counting system. The scintillating fluid contained p-bis- [2-(5-phenyloxazolyl)]-benzene (or POPOP), 0.3 g; 2,5- diphenyloxazole (or PPO), 15 g; toluene, 1140 ml; p-dioxane, 1140 ml; and absolute alcohol, 750 ml.

The necessary amounts of the chelating agent were dissolved in 0.16 M NaCl.

The formation constants for the zinc complexes were calculated from the following expression as derived by Schubert *et al*(6):

$$Y = \frac{(K_d^{\circ}/K_d) - 1}{(A^{-x})} = \frac{}{K_{f(1)} + K_{f(1)}K_{f(2)}(A^{-x})} \quad (5)$$

where K_d is the distribution coefficient for zinc in presence of complexing agent as given by the expression:

$$K_d = \frac{\%\ Zn^{65}\ \text{in exchanger}}{\%\ Zn^{65}\ \text{in solution}} \times \frac{\text{Volume of solution (ml)}}{\text{mass of ion-exchanger (mg)}} \quad (6)$$

As the volume of solution and the mass of ion-exchanger remain constant throughout our experiments,

$$K_d = \frac{\%\ Zn^{65}\ \text{in exchanger}}{\%\ Zn^{65}\ \text{in solution}} \quad (7)$$

and K_d° is distribution coefficient for zinc in the absence of complexing agent of concentration (A^{-x}) moles per liter.

From the expression (5) it can be seen that $Y = K_{f(1)}$ and independent of (A^{-x}) when zinc and the complexing agent form a complex of 1:1 molar ratio. If the numerical values of Y *versus* (A^{-x}) are plotted on a linear graph paper and a line with a finite slope is obtained, then the assumption is that 1:2 complexes also exist. The values of

TABLE I. Formation Constants of Zinc Complexes of Ethylenediaminetetraacetic Acid (EDTA) and Nitrilotriacetic Acid (NTA) by Ion-Exchange Method at a pH of 7.4.

Ligand A, moles	$K_d \pm e$*	$\frac{1}{K_d}$	n	$\frac{K_d^o}{K_d} - 1$	Y†	log formation constant $K_{f(1)}$	log formation constant $K_{f(2)}$
EDTA $\times 10^{-7}$					$\times 10^6$		
0	$2.68 \pm .033$	.37	1				
1.0	$1.84 \pm .077$	.54		.46	4.60		
2.0	$1.34 \pm .22$	.76		1.03	5.15		
3.0	$.76 \pm .25$	1.32		2.52	8.40		
4.0	$.56 \pm .18$	1.78		3.70	9.25	6.49	6.73
NTA $\times 10^{-7}$					$\times 10^{13}$		
2.0	$2.03 \pm .52$	.49		3.92	9.8		
4.0	$.58 \pm .08$	1.73		16.3	10.2		
6.0	$.25 \pm .04$	4.05		39.5	10.95		
0 (extrapolated)		.1	2			13.99	

* Standard error of mean.

† $Y = \frac{(K_d^o/K_d) - 1}{(A^{-x})^n}$.

$K_{f(1)}$ and $K_{f(1)}K_{f(2)}$ can be obtained from the intercept and the slope of this straight line, respectively(6).

Results and discussion. The data for $K_d \pm$ standard error of the mean, $1/K_d$, $(K_d^o/K_d)-1$, and Y for various molar concentrations of EDTA and NTA are presented in Table I to illustrate the calculations of formation constants for their complexes with zinc. When the ligand concentration was zero, K_d^o value was obtained. On plotting the values for Y against the EDTA concentrations, a straight line with a finite slope was obtained. $K_{f(1)}$ value was numerically equal to 6.49, the value of Y corresponding to zero concentration of EDTA.

Since

$$Y = K_{f(1)} + K_{f(1)}K_{f(2)}(A^{-x}) + \ldots \quad (5)$$

The value of $K_{f(1)}K_{f(2)}$ was obtained for EDTA from the slope of the line and

$$\text{Log } K_{f(2)} = \log(K_{f(1)}K_{f(2)}) - \log K_{f(1)} = 6.73 \quad (8)$$

When similar computations were made for the zinc complexes of DHEEDA, HPDTA, NTA, DTPA and EDDADP, the straight lines between the numerical values of Y and (A^{-x}) would not intercept the zero concentration axis. A modified procedure was adapted for calculating formation constants of these complexes and is illustrated by NTA in Table I.

The expression (5) is derived from the general relationship

$$Y = \frac{(K_d^o/K_d) - 1}{(A^{-x})^n} \quad (9)$$

which can be rearranged as follows:

$$\frac{1}{K_d} = \frac{1}{K_d^o} + (A^{-x})^n \frac{Y}{K_d^o} \quad (10)$$

A plot of $1/K_d$ *versus* $(A^{-x})^n$ for proper values of n on a linear graph paper should be a straight line which on extrapolation to $(A^{-x}) = 0$ gives the value of $1/K_d^o$.

A value of 2 was obtained for n for NTA and a value of 13.99 was obtained for log $K_{f(1)}$. A value of $n = 2$ was also obtained for DHEEDA and DTPA; and a value of $n = 3$ was obtained for HPDTA and EDDADP.

The values for formation constants for zinc and various ligands are given in Table II. Citric acid and TPA gave no $K_{f(2)}$ values. The value of 4.53 obtained for zinc-citric acid complex is in reasonable agreement with a value of 4.71 obtained by Schubert *et al* (6) for formation constant of this complex. Also, a value of 1.94 obtained for zinc-glycolate complex is of the same order as the values of 1.81 ($\mu = 0.23$), 1.88 and 1.99 ($\mu = 0.05$) obtained by Schubert *et al* (6); and 1.92 ($\mu = 0.2$) obtained by Cannan and Kilbrick(7).

TABLE II. Formation Constants of Zinc Complexes of Various Complexing Agents by Ion-Exchange Method at a pH of 7.4.

Ligand	n	log formation constant $K_{f(1)}$	$K_{f(2)}$
Terephthalic acid	1	1.00	3.46
Lactic acid	1	1.60	1.90
Thiodipropionic acid	1	1.91	—
Sodium glycolate	1	1.94	2.24
" orthophosphate	1	2.22	1.66
Ethylenediaminebitartrate	1	2.60	1.60
Citric acid	1	4.53	—
Sodium acid pyrophosphate	1	5.27	4.93
" hexametaphosphate	1	5.56	5.46
" tripolyphosphate	1	5.79	4.81
Ethylenediaminediacetic acid (EDDA)	1	5.98	5.15
Hydroxyethylethylenediaminetriacetic acid (HEDTA)	1	6.00	7.40
1,2-Diaminecyclohexanetetraacetic acid (CDTA)	1	6.29	6.69
Dihydroxyethylenediaminediacetic acid (DHEEDA)	2	7.66	
Diethylenetriaminepentaacetic acid (DTPA)	2	12.27	
2-Hydroxypropylenediaminetetraacetic acid (HPDTA)	3	20.08	
Ethylenediamine-N, N'-diacetic acid-N, N'-dipropionic acid (EDDADP)	3	20.91	

No formation constant for zinc-phytic acid complex could be determined by this method because of its insolubility at a pH of 7.4. At this pH, glucose, fructose or lactose did not release Zn^{65} from the ion exchange resin. However, Zn^{65} was released if the pH of the solution was about 10.2.

Summary. Formation constants at a pH of 7.4 and $\mu = 0.16$ have been determined by the ion-exchange method for zinc and a number of complexing agents.

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A Study of Gamma Globulins in Cholera. (30795)

HOWARD B. GOLDSTEIN, BEN N. DALE AND OSCAR FELSENFELD*
Department of Experimental Pathology, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D.C.

The relationship of the various globulins to cholera has not been studied in man with the aid of recently developed methods of chromatographic and electrophoretic separation of serum proteins.

Cholera appears today as a disease caused either by the typical *Vibrio cholerae* or by its biotype, *V. cholerae El Tor*. The latter has been the agent of an epidemic in Asia that involved countries from Indonesia north to Japan and east to Iran since 1961. This so-called El Tor disease is attracting much attention. It seemed advisable, however, to first study "classic" cholera which predominated until 1961 in Asia, reaching out from time to time from its Indian cradle into other countries. The last "classic" cholera epidemic assailed Thailand in 1958 and was the subject of several studies. One of them(1) correlated the agglutinating, lethal toxin and El Tor hemolysin neutralizing potency of the sera of patients with cholera caused by different biotypes of *V. cholerae*. This communication is a continuation of those investigations, reporting the results of the comparison of agglutinating and cholera toxicity neutralizing antibodies with the changes in some im-

* Present address: Tulane Univ., Delta Regional Primate Research Center, Covington, La.