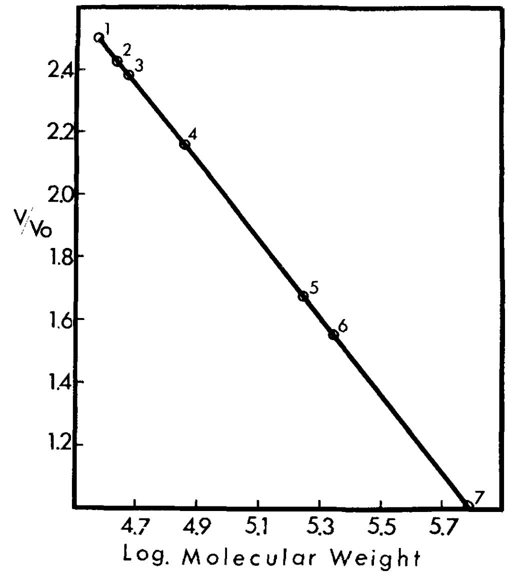

FIG. 1. Relationship between the ratio of the protein elution volume (V) to column void volume (Vo) and the log. of the protein molecular weight. 4°C, pH 5.0, 0.1 M citrate buffer. 1 = pepsin; 2 = human renin; 3 = ovalbumin; 4 = human serum albumin; 5 = bovine gamma globulin; 6 = catalase; 7 = thyroglobulin.

was about 2%. The elution volume of thyroglobulin (m.w. 650,000), that was used to determine the void volume of this system, was 88.2 ml and ranged from 87.8-88.4 ml (S.D. ± 0.28 ml).

The elution volume of renin was determined 4 times. Peak activity of the enzyme was found at an elution volume of 212.8 ml and ranged from 212.5-213.5 ml (S.D. ± 0.41 ml). The molecular weight of human renin as determined by this method was 42,300 (Table I, Fig. 1).

Assuming that this method of molecular weight determination is valid for renin, a close correlation was found between the molecular weights of human renin (m.w. 42,300) as was determined in this study and hog renin (m.w. 42,000-49,000) as determined by Kemp and Rubin(4).

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## Effects of Lanthanide Chlorides on Selected Enzymes. (31637)

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For the past several years the physiological effects of intravenous injection of rare-earth chlorides into rats have been investigated in this laboratory. The most prominent biochemical effect is the reversible development of the fatty liver, which reaches a maximum in 48-72 hours and returns to normal within a week. The increased hepatic fat is essentially due to neutral triglycerides, while phospholipids remain normal(1). Cholesterol and plasmologen levels parallel the increase in neutral lipids(2). In general, female rats appeared to be more susceptible than males but hormonal stresses such as testosterone injection into females, hypophysectomy in both males and females, and adrenalectomy in males can prevent fatty infiltration of the liver(1). This fatty liver is also prevented in susceptible species(3) by chelation (EDTA) or aggregation (hydroxyl ion) of

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the cation before injection(4). Also, glucose affords nutritional protection(5). A probable explanation for this nutritional protection may be the suppression of free fatty acid release from adipose tissue when the blood glucose level is adequate. Some evidence in support of this has been obtained from *in vitro* studies; when glucose is present, a decreased release of nonesterified fatty acids from rat epididymal adipose tissue into the incubation medium occurs(6). Enzymic measurements relating directly to the rare-earth fatty liver show that oxidation of octanoate by liver mitochondria decreases(7).

Although considerable literature is available on the *in vivo* system(1-5,7), *in vitro* systems have been studied less extensively. Therefore, our approach to the problem consisted of a study of the effects of some lanthanides, *in vitro,* on several enzymes important in intermediary metabolism. We selected enzymes from 3 important metabolic pathways: glycolytic cycle (aldolase and lactate dehydrogenases),[†] hexose monophosphate shunt (glucose 6-phosphate dehydrogenase)[†] and the tricarboxylic acid cycle (isocitrate and malate dehydrogenases).[†] An enzyme serving as an important link between amino acid and carbohydrate metabolism (glutamate dehydrogenase)[†] was also chosen. The rare-earth cations selected for this study included lanthanum, cerium, praseodymium, neodymium, gadolinium, dysprosium, holmium, lutetium, and yttrium. Only the first 4 of these are among the lanthanides (atomic numbers 57-62) that cause the fatty liver. The aims we wished to accomplish were 2-fold: the correlation, if any, between lanthanide and heavy metal inhibition of enzyme systems, and a comparison of the effects on selected enzymes of the lanthanides eliciting the fatty liver response with those which do not.

*Materials and methods.* Sources of the materials used in these studies are as follows: rabbit muscle lactate dehydrogenase and malate dehydrogenase from C. F. Boehringer und Soehne; sodium pyruvate, A grade, from the California Corp. for Biochemical Research; sodium D-L isocitrate, α-ketoglutarate, fructose-1, 6-diphosphate, oxaloacetic acid, barium glucose 6-phosphate, and all nucleotides from Sigma Chemical Co.; and sodium glutamate from Nutritional Biochemicals. Barium glucose 6-phosphate was converted to the sodium salt by addition of sodium sulfate and removal of the precipitated barium sulfate by centrifugation. Rare-earth oxides (99.9% purity) were products of Electronic Space Products, Inc., and were converted to the chloride form by dissolving in 6 N hydrochloric acid. Cerium oxide was converted to the chloride according to a procedure published previously(1). Stock solutions of the rare-earth chlorides (0.1 M), which were stored in the dark at pH 1 or below, were diluted appropriately and adjusted to pH 3.5-4 before their use in the enzyme studies. All enzyme experiments were performed in the pH range from 7.2 to 7.5 with the use of Tris-chloride or Tris-maleate as buffer. Stock solutions of all substrates required for the studies, with the exception of DPNH, were prepared, adjusted to neutral pH, and stored at —25°. Aliquots were thawed immediately before use. In all instances, DPNH solution was prepared immediately before use in the kinetic experiments.

Initial reaction velocity was measured either on the Cary 14 recording spectrophotometer or manually with the Beckman DU spectrophotometer by noting the change in absorbency at 340 m$\mu$ of the reduced coenzyme. The molar absorbency index of DPNH was taken to be $6.22 \times 10^3\ M^{-1}cm^{-1}$ (8). Velocity is expressed as the change in the molar concentration of reduced nucleotide per minute. The enzyme, diluted immediately before use, was maintained at 0°. Cuvette temperature was 25°. The enzyme concentration in all instances was adjusted so

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[†] The following abbreviations are used: Lactate dehydrogenase (L-lactate:DPN oxidoreductase, EC 1.1.1.27)-LDH; Glutamate dehydrogenase (L-glutamate:DPN oxidoreductase, deaminating, EC 1.4.1.2)-GDH; Isocitrate dehydrogenase (L$_s$-isocitrate:TPN oxidoreductase, EC 1.1.1.37-ICD; Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate:TPN oxidoreductase, EC 1.1.1.49)-G1-6PD; Malate dehydrogenase (L-malate:DPN oxidoreductase, EC 1.1.1.37)-MDH; Adolase (fructose-1, 6-diphosphate-D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.b); Fructose-1, 6-diphosphate-FDP; Michaelis constant-K$_m$.

TABLE I. Inhibition of Some Dehydrogenases by Heavy Metals.*

| | % Activity | | | | |
|---|---|---|---|---|---|
| | | GDH‡ | | | |
| Cation added | LDH† | F | R | ICD§ | Gl-6-PD‖ |
| None | 100 | 100 | 100 | 100 | 100 |
| Lanthanum | 17 (102) | 73 | 6 (40) | 33 | 9 ( 52) |
| Cerium | 20 (104) | 99 | 9 (77) | 47 | 19 (100) |
| Praseodymium | 22 (106) | 99 | 5 (69) | 79 | 9 (102) |
| Neodymium | 26 ( 96) | 99 | 7 (58) | 37 | 0 ( 25) |
| Gadolinium | 0 (108) | 87 | 8 (54) | 17 | — |
| Dysprosium | 0 (103) | 90 | 7 (58) | 35 | 0 ( 13) |
| Holmium | 0 (116) | 96 | 8 (57) | 25 | 0 ( 0) |
| Lutetium | 0 ( 96) | 99 | 7 (26) | 33 | 0 ( 0) |
| Yttrium | 0 (100) | 90 | 9 (35) | 20 | 0 |
| Copper | 0 | 68 | 7 (87) | 82 | 37 ( 93) |
| Mercury | 16 | 0 | 87 | 55 | 68 (100) |
| Nickel | — | 87 | 6 | — | — |
| Cobalt | — | 82 | 9 | — | — |

* In all instances final reaction volume was 3 ml. Figures in parentheses are percent activity of control in presence of EDTA.

† Reaction mixture samples contained $3.3 \times 10^{-4}$ M pyruvate and $1.5 \times 10^{-4}$ M DPNH in 0.03 M Tris-maleate, pH 7.1. Lanthanide chloride and cupric sulfate concentrations were $8.3 \times 10^{-4}$ M; mercuric chloride concentration was $3.3 \times 10^{-5}$ M. Where indicated, EDTA : metal was 2:1.

‡ In the forward direction substrate concentrations were $3.3 \times 10^{-2}$ M glutamate and $1.0 \times 10^{-4}$ M DPN in 0.03 M Tris-maleate, pH 7.5. Concentration of metal inhibitor was $8.3 \times 10^{-4}$ M. In the reverse direction substrate concentrations were $6.7 \times 10^{-3}$ M $NH_4Cl$, $3.3 \times 10^{-3}$ M alpha-ketoglutarate, and $1.5 \times 10^{-4}$ M DPNH in 0.03 M Tris-maleate buffer, pH 7.5. Concentration of metal inhibitor was $8.3 \times 10^{-4}$ M. Where indicated, equimolar amounts of EDTA and metal were used.

§ Reaction mixture samples contained $3.3 \times 10^{-3}$ M sodium D-L isocitrate, $6.7 \times 10^{-4}$ M $MnCl_2$, $5 \times 10^{-5}$ M TPN, 0.03 M Tris-chloride buffer, pH 7.5, and 0.05 M sodium chloride. Concentrations of lanthanides and copper were $8.3 \times 10^{-4}$ M. Mercuric chloride concentration was $3.3 \times 10^{-6}$ M.

‖ Reaction mixture samples contained $3.3 \times 10^{-4}$ M sodium glucose 6-phosphate and $3.3 \times 10^{-5}$ M TPN in 0.03 M Tris-chloride buffer, pH 7.5. All metals were present at $8.3 \times 10^{-4}$ M, except mercuric chloride, which was $3.3 \times 10^{-5}$ M. Where indicated, equimolar concentrations of EDTA and metal were used.

that the velocity was linear for 3-5 minutes. Alkali-labile phosphate formed in the aldolase reaction was measured by means of Sumner's method(9).

*Results.* One of the problems encountered in investigations of lanthanide inhibition is the formation of highly insoluble compounds such as hydroxides or phosphates. In the present investigation this difficulty was largely overcome by avoiding phosphate buffer and lanthanide concentrations greater than $2.0 \times 10^{-3}$ M. When these conditions were fulfilled, no visible turbidity could be detected in the samples used for the kinetic studies.

The initial studies undertaken in the *in vitro* enzyme systems consisted of a survey of enzyme activity when the rare-earth chlorides enumerated were present. Results are summarized in Table I and reveal that all the lanthanides tested inhibit lactate, isocitrate, and glucose 6-phosphate dehydrogenases. However, the glutamate dehydrogenase reaction is sensitive to the metals tested only in the reverse direction (glutamate formation), while the forward direction is inhibited to a desser degree only by lanthanum, cerium, and mercury. Thus, the differences in response evoked by the various rare-earth salts *in vivo*(1) are not parallel in the enzyme inhibition studies. For comparison with the lanthanides, enzymatic activity measured in the presence of 4 heavy metals is also given. Although these inhibitory effects vary somewhat depending upon the enzyme system studied, the experimental results indicate no reason to treat rare-earth effects as distinct from those caused by some other heavy metals. The figures in parentheses in Table I (columns 1, 3, and 5) correspond to the enzymatic activity in the presence of EDTA.

TABLE II. Cerium Chloride Inhibition of GDH at High and Low Ammonium Chloride Concentrations.

| Cerium concentration ($M \times 10^5$) | $1.67 \times 10^{-4}$ M $NH_4Cl$ | | | $6.67 \times 10^{-3}$ M $NH_4Cl$ | | |
|---|---|---|---|---|---|---|
| | $(NH_4^+)/(Ce^{+3})$ | v* | % Act. | $(NH_4^+)/(Ce^{+3})$ | v* | % Act. |
| 0 | — | .93 | 100 | — | 3.60 | 100 |
| 8.3 | 2 | .48 | 52 | 80 | 1.50 | 42 |
| 25.0 | 0.67 | .24 | 26 | 27 | .73 | 20 |
| 50.0 | 0.33 | .15 | 16 | 13 | .63 | 18 |
| 83.3 | 0.2 | .09 | 10 | 8 | .40 | 11 |
| 167.0 | 0.1 | 0 | 0 | 4 | .26 | 7 |

* Velocity is expressed as molar concentration of DPNH disappearing per minute at 25°.

Experimental conditions are identical except that the EDTA is included along with the metal. In the *in vivo* system, injection of EDTA at a 5.65/1 molar ratio with cerium chloride prevented fat infiltration of the liver (4). It seemed reasonable, therefore, to use EDTA in an attempt to relieve metal inhibition in the *in vitro* enzyme systems under investigation. Isocitrate dehydrogenase was not examined because of the complications arising from the presence of $Mn^{++}$, which is required for enzymatic activity. The ratio of EDTA/metal required to restore full activity is not always the same. Inhibition of lactate dehydrogenase is completely relieved at a ratio of 2 EDTA/1 metal, but the inhibition of glutamate dehydrogenase is only partly relieved. Full activity of glucose 6-phosphate dehydrogenase is restored at a 1/1 molar ratio of EDTA/metal only with cerium and praseodymium. Thus there appear to be differences in enzymatic response when the various lanthanides are examined. Clayton(10) reported similar findings in his studies. The results obtained with glutamate (reverse direction) and glucose 6-phosphate dehydrogenases may be interpreted as indicating either that the affinity of the enzyme for the metal is greater than that of the EDTA or that the metal-EDTA complex is itself binding to the enzyme.

Because of the different requirements of each of the 4 enzymes listed in Table I, further discussion is pertinent. For example, conversion of α-ketoglutarate and DPNH to L-glutamate and DPN requires the presence of ammonium ion. Since it is possible that the metal ion inhibitor could form a complex with ammonia(11), the decreased initial velocity reaction obtained when metal is present could be attributed to removal of $NH_4^+$ through complex formation. That this appears not to be true is evident from Table II in which cerium chloride inhibition of glutamate dehydrogenase is examined at high ($6.67 \times 10^{-3}$ M) and low ($1.67 \times 10^{-4}$ M) ammonium chloride concentrations. Identical experimental conditions were present, except for the ammonium chloride concentration. As the results indicate, the percentage of activity in the presence of the rare-earth chloride is substantially the same, regardless of the $NH_4^+$/metal ratio. This is a strong indication that the removal of ammonium ion through cerium complexing is not occurring. According to Frieden(12) the Michaelis constant ($K_m$) for $NH_4^+$ at pH 8 and 25° is $3.2 \times 10^{-3}$ M. In the results shown in Table II, the low concentration of ammonium ion was 20 times less than the $K_m$, whereas the high ammonium chloride concentration was twice the $K_m$. If cerium were removing some of the $NH_4^+$ by complexing with it, we would expect that a greater amount of inhibition would be obtained at the lower concentration of ammonium chloride, which is below the Michaelis constant for this substrate.

Preliminary results with isocitrate dehydrogenase suggest that the amount of inhibition obtained with a constant concentration of cerium chloride is a function of the manganese/cerium ratio. The Lineweaver-Burk(13) plot shown in Fig. 1 reveals, however, that manganous and cerous ions are not competitive inhibitors; if they were, intersection of the curves on the 1/v axis would result.

The concentration dependence of cerium chloride inhibition was next examined, and the results are summarized in Table III. In all instances substrate concentrations were

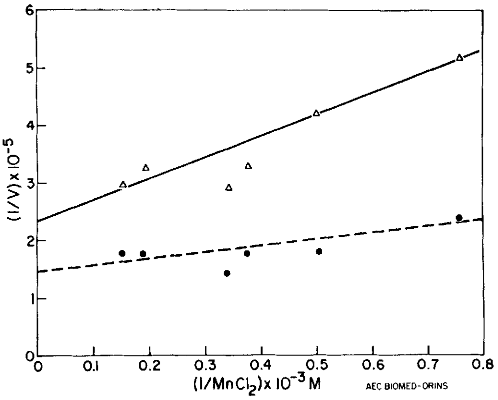

FIG. 1. Plot of reciprocal of initial reaction velocity (v) *versus* reciprocal of the molar concentration of manganous chloride in absence (●) and presence (△) of certain chloride. Reaction mixture samples contained 3.3 x $10^{-3}$ M sodium DL isocitrate, 5 x $10^{-5}$ M TPN, 0.03 M Tris-chloride buffer, pH 7.5, and 0.05 M sodium chloride. Concentration of $MnCl_2$ was varied in the range from 6.7 x $10^{-4}$ M to 5.33 x $10^{-3}$ M; concentration of $CeCl_3$ was 8.3 x $10^{-4}$ M.

such that the enzyme was saturated with substrate. The results again indicate the relative insensitivity of the forward glutamate dehydrogenase reaction to heavy metal inhibition under the experimental conditions used.

In an effort to determine whether more specific information could be obtained concerning the type of rare-earth cation inhibition, a kinetic approach was used. Fig. 2 and 3 show Lineweaver-Burk(13) plots depicting the effects of varying concentrations of cerium chloride on the lactate dehydrogenase reaction. Intersection of the inhibited and uninhibited curves to the left of the 1/velocity axis indicates a "mixed" type of inhibition in which both the maximal velocity and the Michaelis constants are affected by the presence of inhibitor(14). Again, if a purely competitive type of inhibition prevailed (*i.e.*, binding of the lanthanide occurred at a substrate binding site or affected the protein so that the substrate binding ability was decreased), intersection of the inhibited and uninhibited lines on the 1/v axis would result. However, from the experimental results obtained, we can see that a kinetic investigation yields no definite conclusions about the nature of the metal binding. When the reduced coenzyme was preincubated with lactate dehydrogenase before addition of cerium chloride, no protection of enzymatic activity was observed. From the kinetic and substrate-binding experiments we may infer that metal binding is occurring at sites other than the active site, although substrate-binding ability is affected, possibly as a result of the conformational change in the enzyme protein.

Kinetic studies analogous to those shown in Fig. 2 and 3 were carried out with the use of mercuric chloride instead of the lanthanide chloride and gave results similar to those obtained previously. Because mercuric chloride (which is a known sulfhydryl-binding agent) and the lanthanides (which apparently are not specific for the sulfhydryl group) yields similar kinetic results, such an approach cannot be used to gain additional information about the mode of lanthanide binding. Additional evidence that the sulfhydryl group is probably not bound by lanthanide is the fact that cysteine relieves mercurial inhibition but not that of the lanthanides.

Preliminary results obtained with malate dehydrogenase in the direction of malate breakdown suggest that the dehydrogenase is not inhibited by $Ce^{+3}$, $Cu^{+2}$, or $Hg^{+2}$, although the other lanthanides studied produced approximately 50% inhibition at concentrations of 8.3 × $10^{-4}$ M. When inhibition resulted, it was always completely re-

TABLE III. Effect of Cerium Chloride Concentration on Enzyme Inhibition.

| $CeCl_3$ (M × $10^4$) | Velocity* × $10^5$ GDH† (R) | GDH‡ (F) | LDH§ |
|---|---|---|---|
| None | 9.45 (100)* | .76 (100)* | .90 (100)* |
| .83 | 3.10 (33) | .69 (91) | .40 (45) |
| 2.5 | 2.74 (29) | .70 (92) | .23 (26) |
| 5.0 | 1.93 (20) | .69 (91) | .20 (22) |
| 8.3 | 1.09 (11) | .69 (91) | .16 (18) |
| 16.7 | .63 (7) | .66 (87) | 0 |

* Velocity is expressed as the change in the molar concentration of DPNH per minute. Figures in parentheses are percent activity of control.

† Final concentrations of substrates were 6.7 × $10^{-3}$ M $NH_4Cl$, 3.3 × $10^{-3}$ M alpha-ketoglutarate, and 1.5 × $10^{-4}$ M DPNH in 0.03 M Tris-maleate, pH 7.5.

‡ Final concentrations of substrates were 3.3 × $10^{-2}$ M glutamate and 1.0 × $10^{-4}$ M DPN in 0.03 M Tris-maleate, pH 7.5.

§ Final concentrations of substrates were 3.3 × $10^{-4}$ M pyruvate and 1.5 × $10^{-4}$ M DPNH in 0.03 M Tris-maleate, pH 7.2.

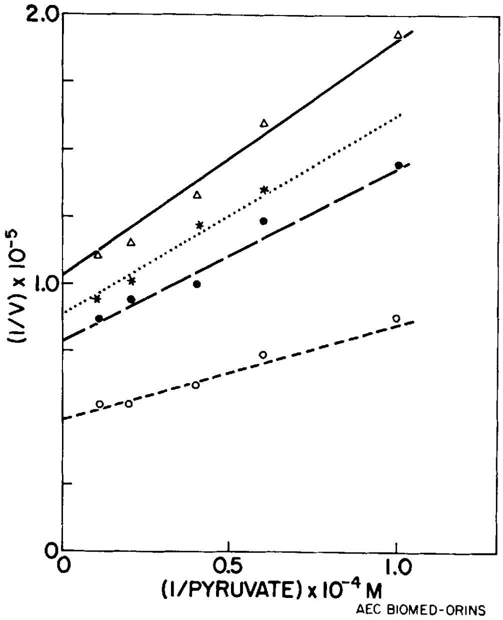

FIG. 2. Plot of reciprocal of initial reaction velocity (v) *versus* reciprocal of the molar concentration of pyruvate in absence and presence of cerium chloride. Concentrations of the lanthanide were none, ○; 2.08 x $10^{-5}$ M, ●; 4.15 x $10^{-5}$ M, *, and 8.30 x $10^{-5}$ M, △. Pyruvate concentration was varied in the range from 1.00 to 8.33 x $10^{-4}$ M, while DPNH was maintained constant at 1.17 x $10^{-4}$ M. Buffer was Tris-maleate, pH 7.2. Velocity is expressed as molar concentration of DPNH disappearing per minute at 25°.

lieved in the presence of an equimolar ratio of EDTA and metal.

Rare-earth chlorides, at a concentration of 1.67 $\times$ $10^{-4}$ M, inhibited the aldolase reaction 50% when fructose-1, 6-diphosphate breakdown was assayed.

*Discussion.* Most of the *in vivo* studies, except some of the initial work(1), have been carried out with cerium chloride. To parallel this work, many of the *in vitro* studies have also used cerium chloride as the inhibitor. Because of the differences in response noted both *in vivo*(1) and *in vitro* (10) when various rare earths were used, no attempt has been made to treat cerium as representative of the lanthanides in their biochemical effects. The effects of the rare earths on the isolated enzyme systems studied cannot be used to explain the *in vivo* observation that some lanthanides elicit a fatty liver response and others do not. In the intact animal the effects of conditions such as the passage of lanthanides across cell membranes, the presence of numerous chelating and binding agents, or the formation of aggregates doubtless play an important role that cannot be assessed from the *in vitro* studies.

The results presented here indicate that lanthanide inhibition of an enzyme appears to be similar in over-all effect to that elicited by other heavy metals such as copper, nickel, or cobalt. Some information was obtained concerning the mode of lanthanide binding. The sulfhydryl group does not appear to be the exclusive site of lanthanide binding. With lactate dehydrogenase, competitive inhibition of substrates and metal is not observed. Furthermore, cerium does not compete with Mn$^{+2}$ in the isocitrate dehydrogenase system nor does it complex with NH$_4^+$ in the glutamate dehydrogenase reaction.

Although only lanthanides with atomic numbers of 57-62 elicited the fatty liver response *in vivo,* parallel results were not observed when single enzymes were examined. All the rare earths tested (atomic numbers 57-71) inhibited enzyme systems sensitive to metal ions. Thus with the data at hand we

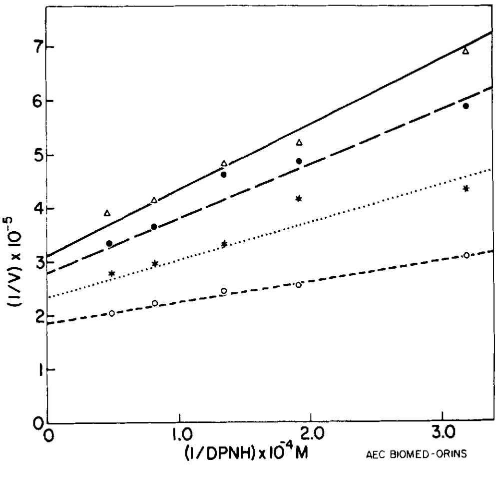

FIG. 3. Plot of reciprocal of initial reaction velocity (v) *versus* reciprocal of the molar concentration of DPNH in absence and presence of cerium chloride. Concentrations of cerium chloride were none, ○; 2.09 x $10^{-5}$ M, *; 8.35 x $10^{-5}$ M, ●; and 1.67 x $10^{-4}$ M, △. DPNH concentration was varied in the range from 0.31 x $10^{-4}$ M to 2.07 x $10^{-4}$ M, while pyruvate concentration was constant at 6.67 x $10^{-4}$ M. Buffer was Tris-maleate, pH 7.2. Velocity expressed as in Fig. 1.

cannot postulate further on the uniqueness of the first 6 rare earths in their biochemical effects.

*Summary.* Lactate, isocitrate, malate, glutamate, and glucose 6-phosphate dehydrogenases and aldolase were examined in the absence and presence of 9 lanthanide chlorides. All these enzymes were inhibited, although not to the same extent. The forward direction of the glutamate dehydrogenase reaction (glutamate deamination) apparently is not sensitive to the lanthanides, although marked inhibition is observed when $\alpha$-ketoglutarate is substrate (reverse direction). The results reveal that all the lanthanides tested (atomic numbers 57-71) can inhibit enzymes sensitive to metals, whereas the fatty-liver response *in vivo* is elicited only by lanthanides with atomic numbers 57-62. The experimental data reveal that lanthanide inhibition *in vitro* is probably a typical heavy-metal effect.

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## Respiratory Excretion of Selenium.[*] (31638)

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The methylation of selenium and the excretion of $(CH_3)_2Se$,(1) in the respiratory gases comprise one of the pathways by which selenium may be eliminated from the animal body(2-6). It appears from the wide range of respiratory values (% dose) reported in the literature that the quantity of selenium exhaled in the respiratory gases increases with the increase in the amount of selenium administered. In order to examine more thoroughly the dose-excretion concept, experiments were performed to determine the correlation between the amounts of selenium administered as either selenite ($^{75}SeO_3^{=}$) or L-seleno-75 methionine, and that excreted in the respiratory gases.

*Materials and methods.* Young adult male rats were injected subcutaneously with a single dose of selenium either as selenite ($^{75}SeO_3^{=}$) or L-seleno-75 methionine (Squibb, Sethotope) to which was added amounts of the correspondent stable selenium compound as indicated in Table I. Following selenium injection, the animals were placed in the Delmar[†] glass metabolism apparatus which permitted the collection of respiratory gases and urine.

A steady stream of dry air was drawn through the system with a water pump, and the expired air was absorbed in a series of 3 gas washing traps. The first 2 traps contained 10% bromine in HBr (V/V) and

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[†] Delmar Scientific Laboratories, Inc., Maywood, Ill.