beginning of a sequence of events which results in encephalomalacia.

The observation that linolenic acid does not promote encephalomalacia would be explained if linolenic acid was not a part of the critical lipid structures involved, or if deposition of this particular fatty acid in the tissues was not increased significantly by its addition to the diet.

Summary. Feeding of heated and aerated unsaturated fats to chickens fed Vit. E low diets resulted in high incidence of encephalomalacia. Incidence of encephalomalacia was correlated with linoleic acid, but not the linolenic acid content of fats fed. Feeding of highly purified linoleic acid resulted in 100% incidence of encephalomalacia. When 20% heated cottonseed oil was the fat source between 44 and 88 I.U./kg of Vit. E or between .0125% and 0.025% of the antioxidant, ethoxyquin was required for prevention of the disease.

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Chelation and Iron Metabolism I. Relative Iron Binding of Chelating Agents and Siderophilin in Serum.* (25627)

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Iron absorption, transport, storage and function are mediated in the organism by the nature of the iron combination. It may be anticipated that knowledge of the factors governing iron complex formation and interaction can lead to new approaches to problems of iron therapy(1,2,3,4). The present study concerns the relative iron-binding strengths of the plasma iron-binding protein siderophilin and various synthetic iron-binding chelating agents in rabbit serum.

Materials and methods. The synthetic chelate compounds, beta hydroxyethyliminodiacetic acid,‡ nitrilotriacetic acid,‡ dibetahydroxyethylglycine,‡ 1,2-propylenediaminetetraacetic acid,§ betahydroxyethylethylenediaminetriacetic acid§ (HOEDTA), ethylenediaminetetraacetic acid‖ (EDTA), and trans-1,

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2-diaminocyclohexanetetraacetic acid,‖ were provided as the pure crystalline products. Stock solutions of the compounds were prepared at a concentration equivalent to an iron-binding strength of 500 γ/ml in appropriate volumes of iron-free water. The solution pH was adjusted to 7.0-8.0 by addition of dilute iron-free sodium hydroxide. For use the stock solutions were diluted to an iron-binding equivalent of 50 γ/ml. Standard stock solutions of iron chelates were prepared by mixing equal volumes of the stock standard chelate solutions and a standard solution of 500 γ/ml of iron prepared by solution of iron wire in dilute hydrochloric acid. The stock standard iron chelate solutions were converted to working solutions at an iron concentration of 50 γ/ml by dilution with iron-free water and pH adjustment to 7.0 immediately prior to use. Serum was prepared from blood obtained by cardiac puncture in the 24-hour fasted rabbit using a siliconized syringe and needle. Determination of the unsaturated iron-binding capacity (UIBC) of an aliquot of each serum sample was carried out by the Ventura modification(5) of the Rath and Finch procedure(6). Division of total optical density change in determination of UIBC by the total gamma of iron added provided a measure for each serum sample of the optical density change for the iron siderophilin formed for a gamma of added iron. This baseline value served as a convenient standard to measure either removal of iron from iron siderophilin or efficiency of formation of the iron protein complex under experimental conditions. The iron chelates utilized in the study showed no absorption in serum at maximum concentrations and wave lengths used in the analyses.

Results. A. *Removal of iron from iron-siderophilin by chelating compounds in serum.* Serum brought to 100% of its iron-binding capacity by addition of increments of standard iron solution was treated with each solution of the enumerated chelate compounds to an equivalent iron-binding of 10-fold the total serum iron content. Other than the minor optical density change resulting from dilution there was no change in absorbancy of the iron protein complex. There was no change in optical density during a 24-hour storage period.

B. *Removal of iron from iron-chelates by siderophilin in serum.* The total amount of iron calculated to be required to saturate UIBC of the serum sample was added in increments in the form of standard iron chelate solutions. Transfer of iron from chelate to iron-binding protein was followed by the increase in optical density due to the iron protein complex. Iron added as the betahydroxyethyliminodiacetic, dibetahydroxyethylglycine and nitrilotriacetic acid chelates was immediately and completely transferred to the protein. Iron added in the form of the other chelates of this study did not increase absorbancy due to the iron protein in an observation period of 12 hours. Typical data for the observed optical density changes are given in Table I.

TABLE I. Comparative Optical Density Data Showing Typical Examples of Results Obtained in Rabbit Serum.

		Optical density at 520 mu		
	Gamma Fe added	Cell 1	Cell 2	Cell 3
1. Fe^{++}	.0	.053	.058	.056
	1.0	.065	.070	.068
	2.0	.077	.082	.080
	3.0	.081	.086	.084
2. Fe-β-hydroxyethylimino-diacetic acid				
	.0	.062	.058	.059
	.9	.072	.067	.069
	1.8	.083	.077	.080
	2.7	.084	.081	.082
	3.6	.084	.081	.082
	12 hr later	.085	.082	.083
3. Fe-ethylenediamine-tetraacetic acid				
	.0	.061	.061	.061
	.9	.062	.061	.061
	1.8	.061	.060	.060
	2.7	.060	.059	.059
	3.6	.059	.058	.058
	12 hr later	.059	.058	.058

C. *Distribution of added iron between siderophilin and synthetic chelates in serum.* Iron was added in one gamma increments to serum samples containing the chelating compounds in quantities equal to twice the total iron-binding capacity of the serum as measured by the UIBC of an aliquot. Under these conditions only HOEDTA demonstrated ability in a 12-hour observation period to bind

‖ Provided by Mr. H. Zussman, Geigy Chemical Co.

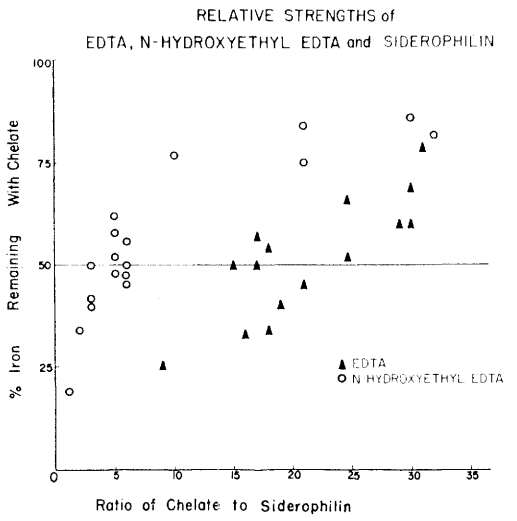

FIG. 1. Distribution of iron added to rabbit serum containing various concentrations of EDTA or N-HOEDTA.

iron in competition with the iron-binding protein of the serum.

The competition between chelate compound and iron-binding protein for iron added to serum was examined for a wide range of chelate/protein equivalent iron-binding ratios for the compounds EDTA and HOEDTA. The results of these measurements are presented in Fig. 1 in terms of percentage of added iron bound by the chelate. It is evident that at the equivalent iron-binding ratio of 3-5/1 for HOEDTA and 15-25/1 for EDTA, distribution of iron added to serum is equal between synthetic chelates and iron-binding protein.

Discussion. Results of this study illustrate problems encountered in application of synthetic metal-binding agents to biological systems. They also provide background information for solution of some of the problems of iron metabolism.

The calculated "stability constant" values provide a useful measure and relative guide of extent of metal-chelate interaction(7). Several experimental conditions are implicit in calculation of these values. Equilibrium reaction conditions, complete ionization of the organic ligand and absence of competing anions or ligands are requisite baseline factors in these measurements. In biologic systems, at the essentially neutral pH range, the kinetics of the reactions of metabolism, distribution and excretion of the metal or the chelating agent may take precedence over and mitigate against attainment of metal-chelate equilibrium.

The present study, using rabbit serum, has equalized the action on all the compounds of the variables of pH, the competitive influence of naturally occurring ligands and anions, and the kinetics of these related reactions on the iron-binding reaction under investigation. In consequence, the effective rather than the theoretical iron-binding ability of the compounds has been determined compared to the iron-binding protein of the blood.

None of the available compounds was able to remove iron from iron-siderophilin in serum. This first indication that synthetic compounds were less effective iron-binding agents than iron-binding protein was not fully substantiated by the next phase of this investigation. While the iron chelates of the ammonia acetic acid series delivered their iron to the protein promptly as might be expected from the first phase of the study, the iron of the iron chelates in the ethylenediamine series was retained by the synthetic compounds. Thus an equilibrium could not be attained in this series in the reaction

Fe siderophilin + chelate $\leftrightarrows$ Fe chelate + siderophilin by approaching from either side of the equation. This result is not entirely unexpected. While the reaction of combination of metal ion and binding agent is usually quite rapid, the exchange reaction of metal ion from one chelate to another can be slow. This is especially the case for exchange of metal between binding compounds of the same relative order of binding strength(8). In an attempt to obviate this kinetic difficulty the study of distribution of added metal ion between competing ligands was performed to gain some insight into the effective iron-binding ability of EDTA and HOEDTA *vis a vis* siderophilin in serum. The results (Section C) indicate that HOEDTA is a somewhat more effective iron-binding agent than EDTA under the conditions present in serum. This finding is in accord with the physical chemical evidence which supports the superiority of the iron-binding ability of HOEDTA in an alkaline medium(9).

Studies of metabolism of parenterally administered EDTA in animals and humans(10, 11) have demonstrated a biological half-life of approximately 30 minutes. When coupled with the present work this would suggest that the synthetic chelates would be limited in iron combination *in vivo* to those areas of the body where they would be in direct competition with the iron-binding protein for "free" ferric or ferrous ions. The tissue iron storage areas are considered to be in this category(12). Here the synthetic chelate could bind iron in competition with the unsaturated iron-binding protein to an extent governed by competitive iron-binding ability, effective compound concentration, size of iron storage, and metabolic fate of the iron chelate.

Summary and conclusions. 1. The iron-binding ability of a series of synthetic chelating agents has been compared to that of siderophilin in rabbit serum. 2. The chelate compounds are unable to remove iron from siderophilin in rabbit serum. 3. Under the same conditions siderophilin is unable to remove iron from the more tightly bound iron chelates. 4. EDTA and HOEDTA, at iron-binding ratios of 20-25 1 and 3-5 1 respectively to that of the protein, compete on equal basis for iron added to rabbit serum.

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Connective Tissue II. Effect of Age and Sex upon Lipid Composition of Tissues of Rat.* (25628)

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Further to correlate the behavior of lipids and protein in connective tissue, the present study was undertaken to determine the effect of age and sex upon: (1) Free cholesterol, (2) Total cholesterol, (3) Phospholipids, and (4) Triglycerides in the connective tissue of various structures of the rat.

Procedures. Male and female rats of the Wistar strain, from 4 weeks to 2½ years old were used in this study. One-tenth to 0.5 g of tail tendon, aorta, skin and uterus, respectively, were cut into small pieces and heated to boiling with 20 ml portions of 1:1 acetone-alcohol, 3 times, and with 3:1 ether-alcohol, twice. After each extraction, the extracting tubes were centrifuged at 2500 rpm for 5 minutes. In each instance, the supernatant was filtered. The combined extracts were evaporated on a steam bath to dryness and made up to a known volume with a 2:1 methyl alcohol-chloroform mixture. Aliquots were taken for determination of free and total cholesterol, phospholipids and total lipids. Free cholesterol was precipitated as digitonide and determined colorimetrically by the method of

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