test provides a relatively specific, though somewhat insensitive system, for detecting gonococcal antibody in human sera. Although it could be a useful adjunct to present laboratory procedures and may have value as an epidemiological tool, it does not provide information concerning the presence or absence of an infection in a human. Current research is directed toward the development of a procedure that will distinguish between present and recent infection.

Summary. An antigen which is suitable for detecting gononoccal antibody is obtained from *Neisseria gonorrhoeae* by phenol extraction. The results of employing this phenol extract antigen in a precipitin test, with 3 groups presumed free of gonococcal infection and 3 groups demonstrated to have gonococcal infection, indicate that it can detect gonococcal antibody in human sera.

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Biliary Excretion of Chelated Iron.* (30618)

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The biliary route is not usually considered a major pathway for physiologic elimination of iron(1,2,3). It can be estimated that in man less than 0.01 mg per day of the daily iron loss arises from bile excretion(4). However, there are some pathologic situations in which the iron content of the bile increases. In hemolytic anemia, for example, the biliary iron in humans increases 5-fold(4). In experimental hemolytic anemia induced by acetylphenylhydrazine, the bile iron in dogs is elevated 10-fold from a normal output of 0.2 mg/day(5).

Our earlier studies provided some suggestion that a suitable chelate ligand could markedly enhance the biliary excretion of parenterally administered iron(6). We have also shown that uptake of iron by the reticulocyte in an *in vitro* system can be sharply modified by the nature of the iron chelate(7). The present study was designed to further investigate, *in vivo*, these possibilities for modification of iron distribution.

Material and methods. Normal adult albino rabbits of both sexes in the weight range of 2-4 kg were maintained on common laboratory food pellets, and water *ad lib.* Food was withdrawn 18 hours before experiments. Following induction of anesthesia with intraperitoneally administered dial-urethane, the abdomen was opened by a midline incision and the hepatic bile duct was isolated by blunt dissection. The duct was cannulated with a

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TABLE I. Ferric Chelates Studied.

Abbreviation	Ligand	Max	R_f*
EDDHA	N,N′ ethylenebis [2-(0-hydroxyphenyl) glycine]	480	.27
CH_3-EDDHA	N,N′ ethylenebis (α imino-2-hydroxy-5-methylphenylacetic acid)	510	.43
Cl-EDDHA	N,N′ ethylenebis (α imino-2-hydroxy-5-chlorophenylacetic acid)	490	.61
SO_3H-EDDHA	N,N′ ethylenebis (α imino-2-hydroxy-5-sulfophenylacetic acid)	474	.0
COOH-EDDHA	N,N′ ethylenebis (α imino-2-hydroxy-5-carboxyphenylacetic acid)	483	.32
DTPA	diethylenetriaminepentaacetic acid		
HOEDTA	N-β hydroxyethylethylenediaminetriacetic acid		
CDTA	1,2-diaminocyclohexane-N,N′-tetraacetic acid	400†	

* TLC silica gel F and butanol-acetic acid-water (90:10:100) visual location.
† F. Bermejo Martinez, R. Rey Mendoza, Chemist-Analyst, 1958, v47, 94.

small bore polyethylene tube which was tied in place. The abdomen was closed and held with clamps. The iron compounds were administered intravenously in the marginal ear vein and for oral studies were injected directly into the intestine at the pylorus. The dosage in all studies was 6 mg Fe/kg. Bile samples were collected at half hourly intervals. Whole blood was obtained by cardiac puncture or by venipuncture sampling from the contralateral marginal ear vein. Approximately 1.5 cc of blood was taken each time. Urine samples were obtained by catheterization or by removal of the bladder at termination of the experiment.

Sprague-Dawley male rats in the weight range of 150-200 g were fasted overnight and their bile ducts ligated while the rat was under a light ether anesthesia. Iron compounds were injected intraperitoneally in the right lower abdominal quadrant and the rats were placed in metabolism cages over glass funnels which were equipped with a cotton plug for separation of urine and feces. At the end of 24 hours, the animals were sacrificed and the urine and a nitric acid digest of the feces and the entire intestine were taken for radioactive counting. Control animals were also anesthetized, sham operated, sacrificed and sampled in the same manner.

The iron chelates (Table I) were prepared by metathesis from ferric chloride and a 20% molar excess of the ligand dissolved in 0.2 M sodium carbonate solution. Iron concentration was 1.4 mg Fe/ml in rabbit studies and for the rats 2.8 mg Fe/ml. Radio ferric chloride ($Fe^{59}Cl_3$)† was added (0.1 μc/ml) and the pH adjusted to 7.0 with sodium carbonate. The solutions so prepared were intensely colored and stable to hydrolysis. Counting was performed in a scintillation well counter using an aliquot of the injected solution as a standard.

The nature of the iron compound in the bile and urine was examined by descending paper chromatography utilizing Whatman No. 1 paper and n-butanol-acetic acid-water (90-10-100). The iron chelates were visually identified by their intense red violet color. The paper strips were counted in a 4 pi strip scanner to establish the locus of radioactivity. For thin layer chromatography, silica gel G‡ and the same solvent system used for the paper chromatography was utilized.

Characterization of the biliary and urinary excretion products was also carried out by spectrophotometric comparison of experimental samples compared to known standards of chelates in control urine and bile collections in the Beckman DU Spectrophotometer. The wave lengths of maximum absorption of the iron chelates are listed in Table I.

Results. Biliary output of Fe^{59} following i.v. administration to rabbits. Fig. 1 presents data obtained for the compounds listed in Table I. The variation for individual animals is small compared to differences in bile excretion as a function of the molecular structure of the iron carrier. The geatest biliary excretion was obtained after N,N′ ethylenebis (α imino-2-hydroxy-5-chlorophenylacetic acid) (Cl-EDDHA), reaching a mean cumulative level of 34.5% of the injected dose by the end of 3 hours. The corresponding methyl homologue, CH_3-EDDHA, was excreted in the bile to the extent of 12.2% in the same

† Abbott Laboratories, Oak Ridge, Tenn.

‡ Research Specialties, Richmond, Calif.

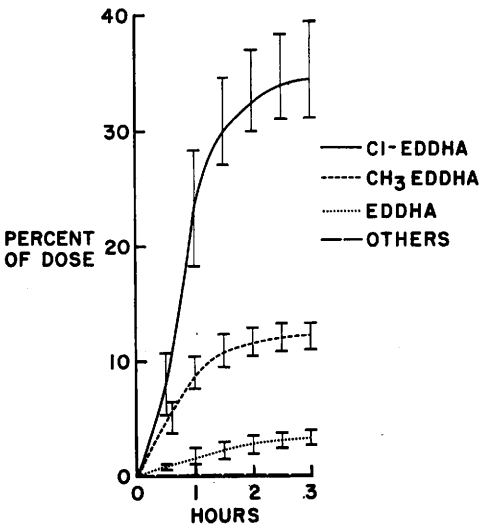


FIG. 1. Bile Fe^{59} after I.V. ferric chelate to rabbits (dose—6 mg Fe/kg). Points represent average values and bars the extremes noted in the experiments.

period. The unsubstituted parent compound, EDDHA, showed a mean 3-hour excretion of 3.3%. Other compounds, Table I, provided minimal evidence of biliary secretion. Less than 0.15% of the administered iron appeared in bile, in each case.

Blood Fe^{59} levels following I.V. iron chelate administration to rabbits. The whole blood Fe^{59} levels concomitant with biliary excretion are illustrated in Fig. 2. The blood disappearance curves do not correlate with the biliary output of iron but rather reflect the summation of other contributory routes of iron distribution. At the end of 1½ hours,

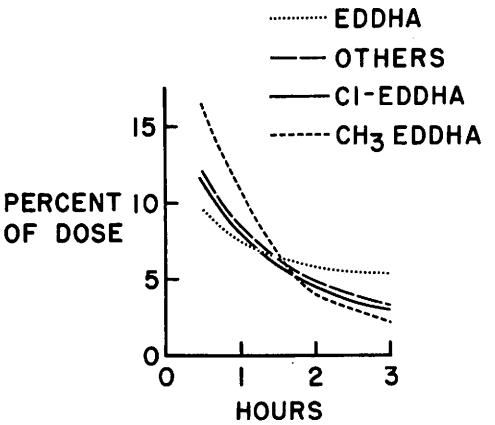


FIG. 2. Blood Fe^{59} after I.V. ferric chelates to rabbits (dose—6 mg Fe/kg).

the circulating radio-iron in the blood is less than 7% of the administered dose for all of the compounds.

Effect of bile duct ligation on Fe^{59} distribution in rats. Fig. 3 summarizes comparative data obtained for iron excretion in feces, GI tract and urine for 24 hours following bile duct ligation and injection of radio-iron chelates. For each of these 3 iron chelates, ligation of the bile duct resulted in enhanced urinary excretion. Despite bile duct ligation the net iron retention was the same as for the non-ligated group.

Iron distribution after oral Fe^{59} chelates to rabbits. Fig. 4, 5, and 6 provide data for biliary, urinary and whole blood iron following oral iron chelate administered to rabbits. The Fe^{59} biliary output (Fig. 4) is highest for Cl-EDDHA followed in order by CH_3-EDDHA, and EDDHA. The other iron chelates, as well as ferric chloride exhibit little biliary iron excretion. Urinary Fe^{59} excretion (Fig. 5), for the compounds of the EDDHA series is in inverse order to their biliary output. EDDHA exhibited an excretion of 9.6%, CH_3-EDDHA was next at 5.9%, and Cl-EDDHA followed at 3.0%. Ferric, 1,2-diaminocyclohexane-N,N'-tetraacetate (CDTA), while not exhibiting any biliary iron excretion showed the highest urinary iron output with excretion of 16.6% of the dose. Blood levels (Fig. 6) are consistent with the results for bile and urine data. The compounds, CDTA, Cl-EDDHA, CH_3-EDDHA and EDDHA which had highest urinary and/or bile excretion also exhibited the highest blood levels. Most rapid absorption was observed for CDTA, followed by the 3 compounds demonstrating biliary excretion, EDDHA and Cl, CH_3-EDDHA. The other compounds exhibited a low and fairly stable blood iron level with a maximum of approximately 1% of the administered dose.

Chemical nature of the iron chelates in vivo. All evidence by spectrophotometry and chromatography (paper and thin layer), is consistent in establishing the radio-iron as a component of undissociated, unaltered iron chelate which was administered. The sequential color of the biliary excretion following oral Cl-EDDHA was most striking. The

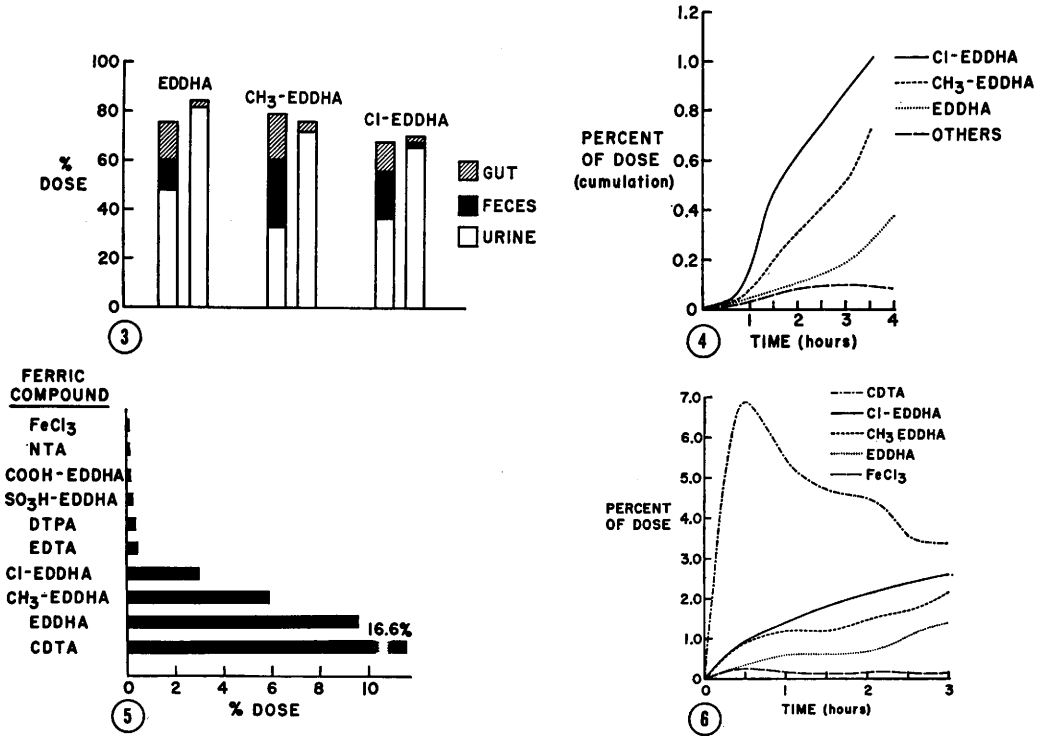


FIG. 3. Fe^{59} distribution after bile duct ligation I.P. (dose—6 mg Fe/kg) to rats 24 hours. Left column presents distribution in the intact animal; right column presents distribution in the bile duct ligated animal.

FIG. 4. Bile Fe^{59} after oral ferric chelate administration to rabbits (dose—6 mg Fe/kg).

FIG. 5. Urine Fe^{59} after oral ferric chelate administration to rabbits (dose—6 mg/kg). After 3 hr.

FIG. 6. Blood Fe^{59} after oral ferric chelate administration to rabbits (dose—6 mg/kg).

characteristic green color of the bile was rapidly changed during the 3-hour observation period to that characteristic of the administered iron chelate. Absorption spectra of the experimental bile samples were identical with control samples to which iron chelate was added and established the identity of excreted iron as the administered chelate.

Discussion. The endogenous iron component of the feces has been shown to arise from two sources. Conrad *et al*(8) have postulated that sloughing of the intestinal epithelial mucosa carries with it a small fraction of endogenous iron. In addition, a minimal amount of iron in the feces arises from the non-reabsorbed fraction of the small amount of bile iron that is normally excreted. The combination of endogenous fecal iron from both these sources does not exceed 0.33-0.52 mg/day in the normal human(4).

While this metabolic pattern is characteristic for naturally occurring iron complexes, sharp alterations in distribution can be induced by modification of the chemical and physical nature of iron through the application of synthetic iron binding agents. Thus it has been possible to induce a high measure of urinary iron excretion in conditions of iron overload by converting normal forms of iron into iron ethylenediaminetetraacetate (Fe EDTA), β-hydroxyethylethylenediaminetriacetate (Fe HOEDTA), diethylenetriaminepentaacetate (Fe DTPA) and desferrioxamine. In these cases, the synthetic iron chelate complexes are excreted in the urine(9-12). These findings have opened new possibilities in treatment of acute and chronic states of iron overload(13,14).

In an earlier report on the influence of synthetic iron binding chelates on iron distribution and excretion, we suggested that some of these compounds provided evidence for a

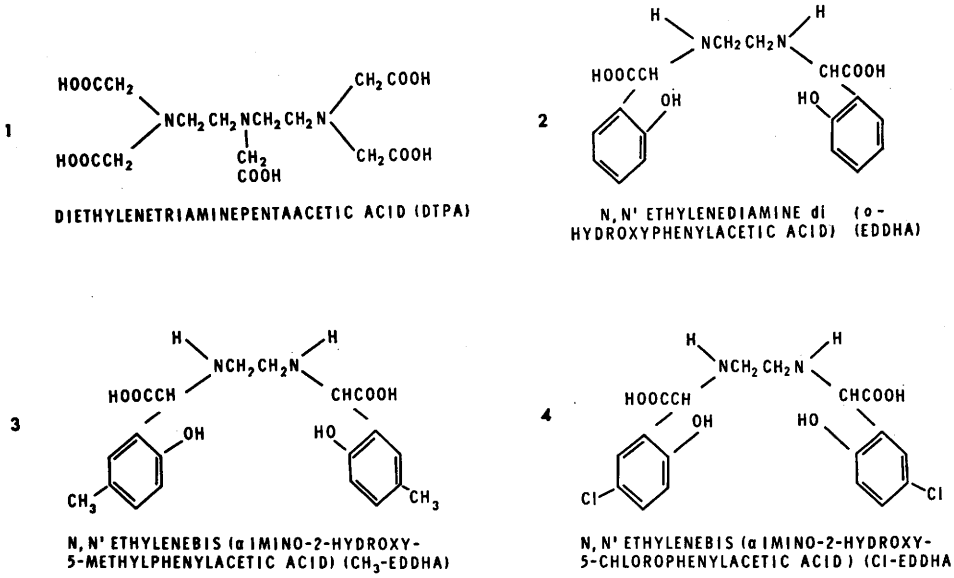
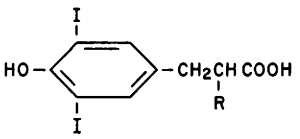


FIG. 7. EDDHA compounds structural formulae. Iron binding occurs at nitrogen, phenolic oxygen and carboxylate sites.

shift of iron excretion into the gastrointestinal tract following parenteral administration(6). While it was not then established experimentally, we suggested that the available evidence indicated that the origin of the intestinal iron was the bile. The present study confirms this hypothesis. Intravenous injection of 5 iron chelates was not followed by significant increase in biliary iron excretion (Table I & Fig. 1). However, ferric EDDHA, CH_3-EDDHA, and Cl-EDDHA demonstrated increasing quantities of biliary iron with a total of 35% of the injected amount recovered in the bile within 3 hours for the last compound (Fig. 1).

An examination of the chemical structures of these 3 iron chelates (Fig. 7, DTPA included for comparison) reveals their relation to the class of iodinated arylaliphatic acids long recognized to exhibit selective excretion by the liver and concentration by the bile (15). These cholecystographic media are characterized by an iodinated benzene ring to which is attached an aliphatic side chain terminating in a carboxyl derivative. The same structure is implicit in EDDHA and its derivatives. It is evident that substitution of the iron atom for the iodine atoms in the radioopaque diagnostic compounds is not an important determinant in the biliary output. We believe that the enhanced biliary iron output for the series of compounds EDDHA, CH_3-EDDHA, Cl-EDDHA represents a gradual shift in their nature toward an increasingly covalently bonded iron structure with intensified lipid character for these iron chelates. Support for this view is provided by the fact that introduction of a charged anionic site into the parent molecular structure brings about a complete disappearance of the ability of the compounds to transport iron into the bile. Thus injection of the iron chelates of SO_3H EDDHA and COOH EDDHA is followed by urinary rather than biliary iron excretion. This is also characteristic of the hydrophilic iron carriers DTPA, CDTA, etc. These results parallel the action of these iron carriers for transport of iron into the reticulocyte(7). The compounds EDDHA, CH_3-EDDHA and Cl-EDDHA which induce biliary iron excretion also enhance iron uptake by the reticulocyte, in the same order. The significant structural features which brought about this result were the net neutral charge and lipid solubility of the iron chelates (7,16). The same factors seem involved in their ability to escort iron into the bile.

In rats as in rabbits, the same compounds following IP injection bring about a high order of fecal and intestinal iron derived from



DIIODOHYDROXYPHENYLALKANOIC ACIDS

FIG. 8. Cholecystographic contrast media.

the bile (Fig. 3). When the bile duct was ligated, the biliary iron was shunted into the urine. It seems clear that these iron chelates have a balanced structure in terms of lipid aqueous distribution which can be directed into either phase. In this respect also the iron compounds are analogous to the iodinated hydroxyphenyl alkanoic acids (Fig. 8). When R = CH_3 or C_2H_5, they were also excreted in the urine rather than in the gall bladder(17). In the series of iron chelates under discussion, the methyl and chloro substituents effectively serve the same function as the increasingly bulky alkyl substituents in the corresponding cholecystographic structures.

Following oral administration to rats, the iron chelates Cl-EDDHA, CH_3-EDDHA and EDDHA were isolated unchanged in the bile. In addition to these compounds, Fe CDTA brought about a considerable urinary iron excretion after oral administration. These facts have interesting implications for the normal mechanism of intestinal iron absorption. It is evident that suitably constituted small molecular weight iron chelates may bypass the usual route of iron absorption. They possibly serve as alternatives for the endogenous small molecular weight iron carriers which have been postulated as a component of transmembrane iron transport(18). It is also not without interest that the combination of absorption *per se* and biliary excretion constitutes an enterohepatic circulating sequence for ferric Cl-EDDHA, CH_3-EDDHA and EDDHA.

Summary. The iron chelates of Cl-EDDHA, CH_3-EDDHA and EDDHA are excreted in the bile unchanged following intravenous and oral administration to rabbits and following intraperitoneal administration to rats. Bile duct ligation results in shunting the excretion into the urine. The metabolic path for these compounds is attributed to their relatively non-polar structure. The absorption of these compounds and Fe CDTA from the intestinal tract without molecular alteration establishes that suitably constructed small molecular weight iron chelates may bypass physiologic mechanisms of oral iron absorption.

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