riftia tube worms, galatheid crabs, and *Calyptogena magnifica* clam, as well as other organisms, are present at the Galápagos site and the East Pacific Rise hydrothermal vents at 21°N. The mechanism by which these benthic organisms migrate from one isolated deep-sea hydrothermal vent site to another is likely to be in the form of a planktonic larval stage (*26*).

The most commonly observed and morphologically conspicuous microorganisms found at the Galápagos hydrothermal vent systems have been described (*27*). These microorganisms and those found at the East Pacific Rise participate in a diverse number of microbial processes; they include sulfur-oxidizing, metal-oxidizing, and methane- and methylamine-oxidizing bacteria, as well as the thermophilic methanogen *M. jannaschii* (*28*). Since the macrocycle **1a** appears to occur only in *M. jannaschii*, this molecule can potentially be used as a species-specific marker to assess this microbe's contribution to the archaebacterial community in the hydrothermal vent systems. In addition, the use of this molecular marker and the other glycerol ethers should enable us to determine whether the archaebacterial community is relatively homogeneous at geographically distant and isolated hydrothermal vent sites. Our screening does not preclude the possibility that other hydrothermal vent archaebacteria biosynthesize the macrocyclic ether **1a**, but certainly the absence of **1a** among the glycerol ethers in hydrothermal vent organic material would rule out the presence of *M. jannaschii*.

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The Exclusion of D_2O from the Hydration Sphere of $FeSO_4 \cdot 7H_2O$ Oxidized by *Thiobacillus ferrooxidans*

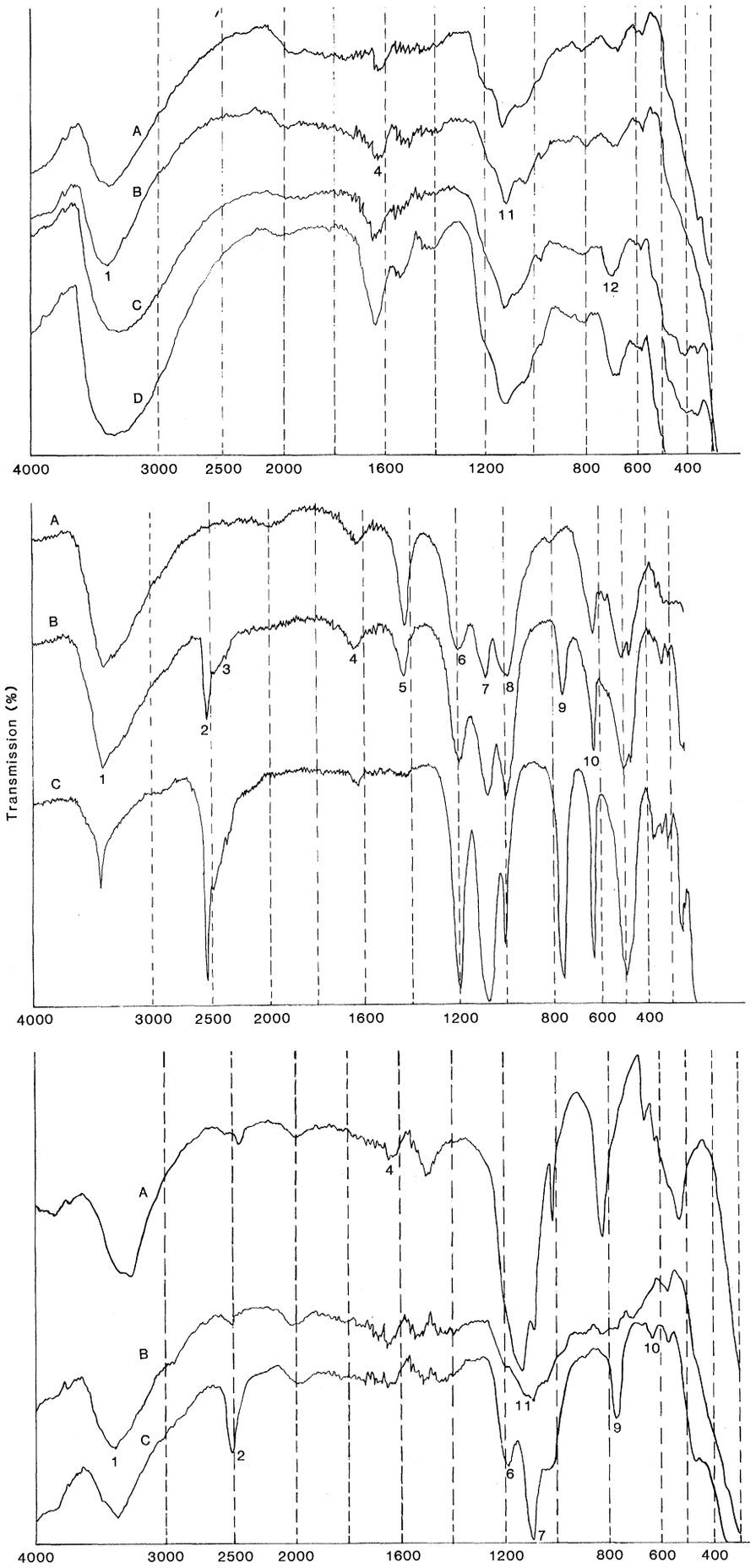
Abstract. *Infrared spectra demonstrate that neither $FeSO_4 \cdot 7H_2O$ nor its bacterial or abiotic hydrated oxidation products incorporate deuterium in acid D_2O solutions. Deuterium exchange occurred as bridging OD when bacterially oxidized iron was precipitated from D_2O solutions as ferric hydroxysulfates. The exclusion of deuterium depended upon the stabilization of aquated Fe(II) and Fe(III) complexes by sulfate ions in outer-sphere coordination and is consistent with the requirement and postulated role of sulfate in iron oxidation by* Thiobacillus ferrooxidans.

Despite the recognized importance of metal ions to living systems, often overlooked is the fact that all biologically important cations are present in aqueous solution as hydrated species. The chemical reactivity of a cation may depend upon molecular exchange between its primary hydration sphere and other molecules in solution (*1*), yet very little is known about how the biological activity of a metal ion relates to the coordinated water molecules. I present evidence that oxidation of iron by a chemolithotrophic bacterium is dependent upon the existence in acid solution of a Fe(II) hydrate complex stabilized by SO_4^{2-}. The organism, *Thiobacillus ferrooxidans*, is important in the natural leaching of pyritic minerals and in biohydrometallurgical technology (*2*).

Although rates for the exchange between solvent water molecules and water molecules in the hydration sphere of aquated ions differ widely, even the most tightly bound are reported to exchange at measurable rates (*1, 3*). For example, one of the slowest reported is hexaaquated Rh(III), in solution with perchlorate ions ($t_{1/2}$ = 33 hours) (*4*); more common are values like 10^{-6} second for hexaaquated Fe(II) ammonium sulfate or hexaaquated Co(II) (*5*).

A slower range of exchange rates would be expected with D_2O as solvent than with H_2O, since the energy required for breaking deuterium bonds is generally thought to be greater than for hydrogen bonds (*6*). However, isotope effects alone would not prevent the formation of deuterated complexes, at measurable rates, during incubation of soluble hydrated complexes in 95 percent D_2O solutions (*7*). It was extraordinary, therefore, to find that hydrated Fe(III) precipitates derived from $FeSO_4 \cdot 7H_2O$ in 95 percent D_2O solutions, acidified to permit oxidation by *T. ferrooxidans*, did not contain demonstrable amounts of deuterium.

Earlier investigation had shown that oxidation by the acidophilic chemolithotrophic bacterium *T. ferrooxidans* precipitated an amorphous hydrated Fe(III) sulfate from acidic Fe(II) sulfate solutions (*8*). This precipitate was similar in composition and infrared spectrum to an amorphous Fe(III) sulfate ($2Fe_2O_3 \cdot SO_3 \cdot mH_2O$) described by Margulis *et al.* (*9*). In an effort to characterize this material and determine if the



precipitates contained bridging OH groups, I carried out the oxidation of $FeSO_4 \cdot 7H_2O$ in acidified D_2O solution. Although the products of bacterial oxidation were of particular interest, infrared spectroscopic comparisons were made of sediments obtained from abiotically as well as bacterially oxidized iron in acid D_2O or H_2O solutions (Fig. 1).

The protocol for bacterial oxidation consisted of incubating 20 ml of 0.1*M* $FeSO_4 \cdot 7H_2O$ in H_2O or 95 percent D_2O, containing 3.0×10^{10} washed cells of the Leathen strain of *T. ferrooxidans*. The *p*H of the solution was adjusted with concentrated H_2SO_4 to between 2.2 and 2.5, corresponding to a *p*D range between 2.6 and 2.9 in D_2O solutions (*10*). Approximately 21 percent of the iron in D_2O solutions was oxidized by the bacterial suspension in 30 hours of continuous shaking at 20°C. In that time, the amount of iron oxidized in D_2O was 68 percent of that in H_2O. After 48 hours, sufficient hydrated Fe(III) sulfate had precipitated to be recovered for analysis (*11*). After the first crop of precipitate had been removed by centrifugation, supernatant

Fig. 1 (top). Infrared spectra of amorphous hydrated Fe(III) sulfate precipitates obtained after oxidation of 0.1*M* $FeSO_4 \cdot 7H_2O$: (A) by H_2O_2 in 95 percent D_2O at *p*D 2.9; (B) by heating to 80°C in D_2O at *p*D 2.9; (C) by cells of *Thiobacillus ferrooxidans* in H_2O at *p*H 2.5; and (D) by cells of *Thiobacillus ferrooxidans* in 95 percent D_2O at *p*D 2.6. Similar spectra were obtained for precipitates recovered over a 14-month period. The numbering is explained in Fig. 3. Fig. 2 (center). Infrared spectra of ammoniojarosites, formed by the addition of solid $(NH_4)_2SO_4$ to solutions remaining after the removal of bacteria and amorphous precipitates from 0.1*M* $FeSO_4 \cdot 7H_2O$, oxidized by *Thiobacillus ferrooxidans*: (A) in H_2O at *p*H 2.5; (B) in D_2O at *p*D 2.6, 12 days after addition of 0.2*M* $(NH_4)_2SO_4$; and (C) in D_2O at *p*D 2.6, 14 months after removal of the initial precipitate of ammoniojarosite (B). Fig. 3 (bottom). Infrared spectra of Fe(II) and Fe(III) sulfates formed from $FeSO_4 \cdot 7H_2O$ in 95 percent D_2O: (A) Spectrum of $FeSO_4 \cdot H_2O$ formed by the evaporation of $FeSO_4 \cdot 7H_2O$ solution in 95 percent D_2O at *p*D 2.6. (B) Spectrum of predominantly amorphous hydrated Fe(III) sulfate with small amounts of sodium jarosite; recovered after the addition of 0.1*M* solid Na_2SO_4 to a filtered solution of bacterially oxidized $FeSO_4 \cdot 7H_2O$ in 95 percent D_2O. (C) Spectrum of sodium ferric hydroxysulfate, recovered after the addition of 0.2*M* solid NaCl to a filtered solution of bacterially oxidized $FeSO_4 \cdot 7H_2O$ in 95 percent D_2O. The infrared band assignments (*13*, *19*) are as follows: *1*, OH stretch, with NH stretch shoulder in ammoniojarosite; *2*, OD stretch; *3*, ND stretch, shoulder on OD stretch of ammoniojarosite; *4*, H_2O deformation; *5*, NH deformation; *6*, ν_3, $(C_{3v})SO_4^{2-}$; *7*, ν_3, $(C_{3v})SO_4^{2-}$; *8*, δ_{OH} (bridging); *9*, δ_{OD} (bridging); *10*, ν_4, SO_4^{2-}; *11*, ν_3, $(T_d)SO_4^{2-}$; *12*, ν_r (hindered rotation), H_2O.

solutions were passed through 0.22-μm filters to remove bacterial cells and terminate oxidation. Amorphous Fe(III) sediments that continued to form in the bacteria-free D_2O filtrates, over a period of 14 months, lacked deuterium and were uniform in infrared spectral characteristics (Fig. 1).

Abiotic oxidation of $FeSO_4 \cdot 7H_2O$ in acid D_2O solutions, accomplished either by heating to 80°C or by the dropwise addition of 30 percent H_2O_2, caused rapid precipitation of Fe(III) as an amorphous hydrated sulfate similar to that formed by bacterial oxidation. This was contrary to earlier findings (*6*), which I again confirmed, that abiotic oxidation in H_2O produced precipitates with infrared bands indicative of admixture with αFeOOH. However, D_2O sediments formed after abiotic oxidation lacked the distinctive librational OH bands of αFeOOH and bands due to deuterium incorporation (Fig. 1).

In contrast, when filtrates of supernatant solutions, derived from bacterial oxidation of iron in D_2O, were treated with solid $(NH_4)_2SO_4$ to form ammoniojarosite, $(NH_4)Fe_3(SO_4)_2(OH)_6$, deuterium incorporation did occur as evidenced by strong OD stretch, ND stretch, and OD bridging frequencies (Fig. 2). Continued precipitation of ammoniojarosite from D_2O produced sediments with more complete replacement of protium by deuterium (Fig. 2C).

Sodium ions are not as efficient as monovalent cations of larger radii in forming jarosites in bacterially oxidized iron solutions (*7, 12*). A D_2O solution of bacterially oxidized $FeSO_4 \cdot 7H_2O$, treated with solid sodium sulfate, produced a brown precipitate that consisted mainly of the amorphous hydrated Fe(III) sulfate, as shown by its fibroporous microstructure under scanning electron microscopy. This was confirmed by infrared spectroscopy (Fig. 3) and sodium analysis, which showed that the material contained very little deuterium or sodium jarosite. In contrast, the addition of solid NaCl to bacterially oxidized iron in D_2O solution yielded a yellowish precipitate that had the infrared spectrum of a ferric hydroxysulfate (Fig. 3C), with OD present instead of bridging OH (Fig. 3). This result was unexpected because the addition of chloride ions to H_2O solutions of bacterially oxidized Fe(III) sulfates generally had yielded amorphous sediments rather than crystalline Fe(III) hydroxysulfates (*8*). Scanning electron microscopy, x-ray diffraction, and flame photometric analysis indicated that the substance was a crystalline sodium ferric hydroxysulfate similar to natrojarosite. However, the amount of iron in the precipitate (~ 26 percent) was less than expected for natrojarosite.

The absence of deuterium exchange, either during or after oxidation of $FeSO_4 \cdot 7H_2O$, indicated that the hydration spheres of both the Fe(II) and Fe(III) complexes were unexpectedly stable in the acid D_2O systems that I studied. This result suggested that outer-sphere SO_4^{2-} [associated with Fe(II) or Fe(III) complexes] not only prevented the replacement of H_2O molecules by D_2O but also prevented the exchange of deuterons and OD^- with protons and OH^- that arise from dissociation of water. This interpretation was corroborated by infrared spectra of $FeSO_4 \cdot H_2O$, formed by forced-draft evaporation of $FeSO_4 \cdot 7H_2O$ in acid D_2O solution. The infrared spectrum of the monohydrate formed (Fig. 3A) after several months of equilibration of the heptahydrate in D_2O at room temperature, showed very little deuterium present. This recalcitrance to deuterium exchange persisted despite changes in the coordination sphere of Fe(II) during conversion of the heptahydrate to the monohydrate, particularly, the introduction of outer-sphere (T_d) SO_4^{2-} into an inner coordination site, as shown by its conversion to C_{3v} symmetry (*13*).

Earlier reports of SO_4^{2-} stabilizing the coordinated water of Fe(II) hydrates (*13, 14*) and other hydrates of divalent cations (*13, 15*) give no indication that the stabilities noted would have the effect of excluding solvent exchange. However, one may suppose that deuterium exclusion signifies the existence of an exchange barrier greater than that usually attributed to a deuterium isotope effect (that is, due to the strength of deuterium bonding between heavy water molecules in the solvent phase and their reduced mobility as compared to that of H_2O).

The role of SO_4^{2-} in stabilizing aquated Fe(II) and Fe(III) complexes in acid solution parallels the requirement for SO_4^{2-} in iron oxidation by *T. ferrooxidans* (*16*). Although this requirement is best satisfied by high concentrations of SO_4^{2-}, other oxyanions such as PO_4^{3-}, AsO_4^{3-}, WO_4^{2-}, and TeO_4^{2-} partially replace SO_4^{2-} and SeO_4^{2-} replaces it completely (*17*). The loose specificity of the bacterial requirement can now be understood to mean that *T. ferrooxidans* needs an oxyanion-stabilized hexaaquated complex of Fe(II) as substrate for iron oxidation rather than a particular anion at high concentration.

Conceivably, oxyanions stabilize hydrates by hydrogen bonds which bridge coordinated water molecules of the iron complex (*18*). In contrast, anions such as chloride or nitrate, which inhibit iron oxidation by *T. ferrooxidans*, destabilize the required substrate complex by replacing SO_4^{2-} in the outer sphere. Such action would destructure the hydration sphere and lead to replacement of water molecules by other ligands. Similarly, cations suitable for interaction with the Fe(III) complex can release stabilizing hydrogen bonds and allow entry of bridging SO_4^{2-} and OH^- into the complex to replace water molecules and form jarosites.

In nature, iron and SO_4^{2-} occur together in acid solution wherever pyritic minerals are exposed to air and water. The reduced sulfides serve as reservoirs of chemical energy for the iron-oxidizing thiobacilli (*2*). As the iron sulfide minerals are leached by bacterial action, an aqueous environment results that contains, as major solutes, hydrated Fe(II) and Fe(III) with excess SO_4^{2-}. The association of iron and sulfur is maintained in such environments by the activity of organisms such as *T. ferrooxidans*, which coprecipitate both elements by oxidizing Fe(II) in hydrated complexes stabilized by SO_4^{2-}.

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Estradiol Fatty Acid Esters Occur Naturally in Human Blood

Abstract. *Treatment of nonpolar ether extracts of human female blood with mild alkali produced more immunoassayable estradiol than the unhydrolyzed extract. Analysis of the serum extracts showed that the substance which released immunoreactive estradiol after hydrolysis has chromatographic properties identical to those of fatty acid esters of estradiol esterified at carbon 17. The physiological role of these previously unknown endogenous esters might be inferred from their structural similarity to synthetic drugs used therapeutically for their prolonged estrogenic action.*

For over a half-century, alkyl and aryl esters of estradiol have been known as biologically potent steroids, generally considered to be "long-acting" estrogens (*1–3*). These esters, such as estradiol benzoate, propionate, cyclopentylpropionate, and valerate are synthetic drugs still used for prolonged estrogen stimulation (*4–6*). The most potent natural steroidal estrogen, 17β-estradiol (E_2), exists not only as the "free" steroid, but in conjugated form. However, the conjugates are exclusively ionic, extremely water-soluble polar steroids linked to glucuronic acid or sulfate moieties (*7, 8*). To our knowledge, the natural occurrence of estrogenic steroids similar to the pharmacologically active, nonpolar alkyl esters of estradiol has not previously been shown. We now present evidence that alkyl esters of estradiol normally circulate in human blood and thus exist endogenously in nature.

An unusual nonpolar metabolite of estradiol was produced in our laboratory by in vitro incubations of several tissues (*9*). This compound, which could be converted back into estradiol by mild hydrolytic treatment, was named the lipoidal derivative of estradiol (LE_2) to underscore its unique hydrophobic nature and uncertain structure. Subsequently, we isolated and identified LE_2 as a family of fatty acid esters of estradiol esterified exclusively at carbon 17 (*10*). The experiments described here were designed to determine whether LE_2 is present in human blood. Using immunochemical and chromatographic techniques, we found that a nonpolar compound with properties identical to those of LE_2, the carbon 17 esters of estradiol, exists in serum.

We reasoned that if LE_2 is present in human blood, mild alkaline hydrolysis of an ether extract of serum should produce more estradiol than the untreated extract. Unlike LE_2, other known conjugates of estradiol, such as sulfates and glucuronides, are neither extractable with ether (*11*) nor hydrolyzable with a weak base (*12*). Estradiol was measured by radioimmunoassay with an antiserum highly specific for estradiol (*13*). Esters representative of LE_2, such as estradiol-17-stearate and estradiol-17-arachidonate, did not cross-react in this assay. Serum samples (*14*) from males, cycling

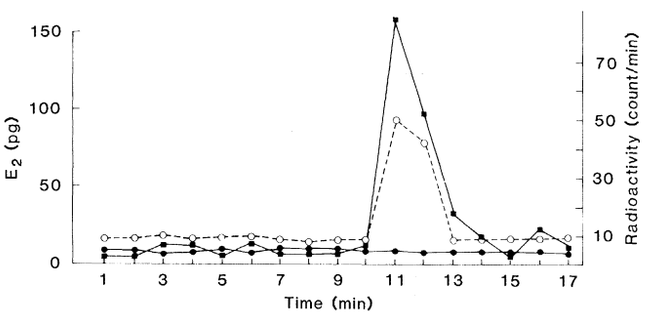

Fig. 1 (left). High-pressure liquid chromatography of the LE_2 fraction from human serum. A serum sample (2.0 ml) obtained from an HMG-treated subject was extracted with ether after [^{3}H]estradiol-17-stearate (1000 count/min) had been added. The ether was evaporated and the residue was purified on a silica gel column and by HPLC (Table 2). Fractions of 1 ml were collected and 200-μl portions were counted for tritium. One half of the remainder of each fraction was analyzed for estradiol by radioimmunoassay in duplicate. The remaining half of each fraction was hydrolyzed overnight in alkali. Estradiol was radioimmunoassayed in duplicate in these hydrolyzed fractions. The control values were obtained from the first four fractions, which elute from the HPLC column before the solvent front. The E_2 values shown are those obtained after subtraction of the controls (< 10 pg before and 79 pg after hydrolysis). Symbols: (○) tritium, (●) E_2, and (■) E_2 after alkaline hydrolysis.

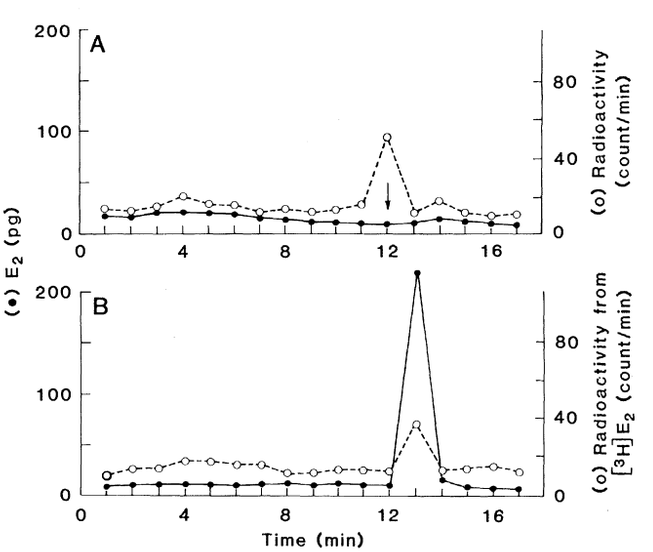

Fig. 2 (right). (A) High-pressure liquid chromatography of LE_2 fraction isolated from human serum. Tritiated estradiol-17-stearate was added to serum obtained from a patient receiving HMG. LE_2 was extracted and purified by silica gel chromatography and HPLC (Fig. 1). Fractions of 1 ml were collected and tritium was counted in 100-μl portions. The remainders of each fraction and one fifth of fraction 12 (arrow) were analyzed for E_2 by radioimmunoassaying duplicate portions. The control value for the radioimmunoassay, 13 pg per fraction, has not been subtracted. The quantity of E_2 in fraction 12 has been normalized to correct for the size of the portion tested. (B) High-pressure liquid chromatography of K_2CO_3-treated fraction 12. Fraction 12 of the HPLC [arrow in (A)] in which [^{3}H]estradiol-17-stearate migrated was evaporated and hydrolyzed overnight with K_2CO_3. The alkali-treated fraction was processed in the usual manner and purified by HPLC on a LiChrosorb Diol column with methylene chloride at 1 ml/min. Fractions of 1 ml were collected. Portions of 100 μl were counted for detection of [^{3}H]estradiol. The remainder of each fraction was analyzed for estradiol by radioimmunoassaying duplicate portions. The control value was < 10 pg.