is of the same duration as the period used for conventionalization of ex-germfree mice. The number of coliform bacteria in the stools of monoinfected mice is of the same order or even higher than in conventional mice or conventionalized ex-germfree mice. The same has been found to be true when *E. coli* is superimposed on the intestinal flora of SPF mice kept in isolators.

Other factors obviously play a role. One might suggest that other microbial products could affect the resorption of bacterial lipopolysaccharides from the intestinal tract and thus indirectly contribute to the induction of susceptibility primarily inflicted by the lipopolysaccharides.

Summary. Germfree mice of the NIH and Lobund strains, as well as specific pathogen-free mice, are significantly more resistant to the lethal effect of endotoxin, derived from *E. coli* strains indigenous to the mouse, than conventional mice of the same genetic stock. Conventional NIH mice were generally more susceptible to *E. coli* endotoxin than conventional Lobund mice. The NIH conventional mice harbored 10-100 times more coliform bacteria in their intestinal contents than the Lobund conventional mice. Differences in the gram-positive flora were also noted. Whereas conventionalization of germfree mice or SPF mice enhanced their susceptibility to endotoxin, contamination of germfree mice or SPF mice with single strains of *E. coli* failed to increase susceptibility to endotoxin.

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A Micromethod for Determination of Acidic Substances of Biological Significance. (28470)

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Currently available methods for determination of minor acidic components of plasma and other biologic material, including free fatty acids (FFA) and bile acids (conjugated or free) may not be sufficiently specific and sensitive at the micro level. We wish to report preliminary studies utilizing (a) the quantitative methylating properties of diazomethane, (b) the excellent fractionation capabilities of thin-layer chromatography and (c) the extreme sensitivity of radioactivity determinations following introduction of C^{14}-methyl groups of high specific activity.

Diazomethane is known to react with compounds containing a variety of structures, but the reaction with carboxylic acids to form methyl esters occurs more readily and under milder conditions than with most other groups(1). Thin-layer chromatography on silicic acid offers excellent prospects of isolating the methyl esters formed. When such esters are radioactive, they may be eluted from silicic acid and counted. Provided methylation is complete, the amount of radioactivity will be directly proportional to the amount of acid originally present. Absolute

amounts may be calculated by comparison with known amounts of standard acids, methylated and chromatographed under the same conditions.

Methods. Plasma lipids were extracted and washed by the method of Folch *et al.*(2). Free fatty acids in the extracted lipids were separated by the use of basic ion-exchange resin. A column, 5 × 1 cm, of Dowex AG1-x2 resin was prepared in hydroxy form by treatment with 20 ml of 2N NaOH followed by thorough washing with CO_2-free water and then with wet diethyl ether. Lipids from 1 ml normal plasma were applied in ether and acid-free material was eluted with 50 ml wet ether. Extracts of saponified bile were prepared by the method of Levin *et al.*(3). Fatty acids and bile acids were obtained from *Calbiochem* (Los Angeles).

An ethereal solution of diazomethane was prepared(4) by alkaline treatment and distillation of 200 mg of N-methyl-N-nitroso-*p*-toluene sulfonamide (New England Nuclear Corp.) containing 20 μc of N-$C^{14}H_3$ and stored in a freezer until use. All operations with it were conducted in a fume hood.

For thin-layer chromatography, Pyrex glass plates (20 × 20 cm) were coated with Silica Gel G and activated at 110° for 3 hours. Following application of lipids, the plates were developed in mixtures of organic solvents and the spots were visualized by heating the plates at 250° after spraying them with 50% (v/v) sulfuric acid. To locate the radioactive spots, standard methyl esters were employed as markers which were visualized in iodine vapor after masking the active spots with another glass plate. Silicic acid from the areas bearing active material was scraped off, transferred to small columns and eluted with diethyl ether into counting vials. The eluates were evaported to dryness and the radioactivity determined in a (Tri-Carb) liquid scintillation spectrometer(5) to give a standard deviation of less than 2%.

To obtain autoradiograms, the developed plates were left in contact with X-ray films for 3 weeks.

Results. Completeness of methylation. The extent of reaction of diazomethane with fatty acids and bile acids was studied. The materials (Fig. 1 and 2) were dissolved in about 100 μl of diethyl ether containing one drop of methanol, and then treated with 150 μl of ethereal diazomethane at about 20°C for 30 minutes in the dark. The solvent and excess diazomethane were removed with a stream of nitrogen and the residue transferred in chloroform to single spots on thin-layer plates. Fatty acids and their esters (Fig. 1) were run in a mixture of petroleum ether; diethyl ether; glacial acetic acid; 90:10:1 v/v (Solvent 1) and bile acids (Fig. 2) in 80:20 chloroform-methanol (Solvent 2). It is apparent that methylation of standard FFA and those present in plasma, as well as standard bile acids and those present in saponified bile, was complete.

Radioactive products. Methylation and chromatography, as previously described, (Solvent 1) were carried out using C^{14}-diazomethane (150 μl) in the absence of any lipids. Autoradiograms were made of this plate and the plates shown in Fig. 1 and 2; and these are represented diagrammatically in Fig. 3. They indicate the presence of radioactivity, not only in methyl esters of the acids but also in other areas. This radioactivity was seen between the origin and the position occupied by cholesterol in Solvent 1, and close to the solvent front in Solvent 2. The major part of this activity was apparently derived from diazomethane, since it was also present in the absence of lipids.

Quantitation. Mixtures containing free fatty acids or cholic acid in microgram quantities as shown in Tables I and II were methylated with C^{14}-diazomethane, the esters separated by thin-layer chromatography in a system containing petroleum ether; diethyl ether; glacial acetic acid 97:3:1 (Solvent 3, for fatty acids); or 90:10 chloroform-methanol (Solvent 4, for bile acids). Methyl esters were eluted and counted. The results of 3 typical experiments are given in Tables Ia, Ib, and II. They indicated that (1) the activity recovered was proportional to the amounts of fatty acids and cholic acid present and (2) other components of plasma lipids caused no interferences in the recovery of the radioactivity in methyl esters of the acids. The FFA content of a test plasma was

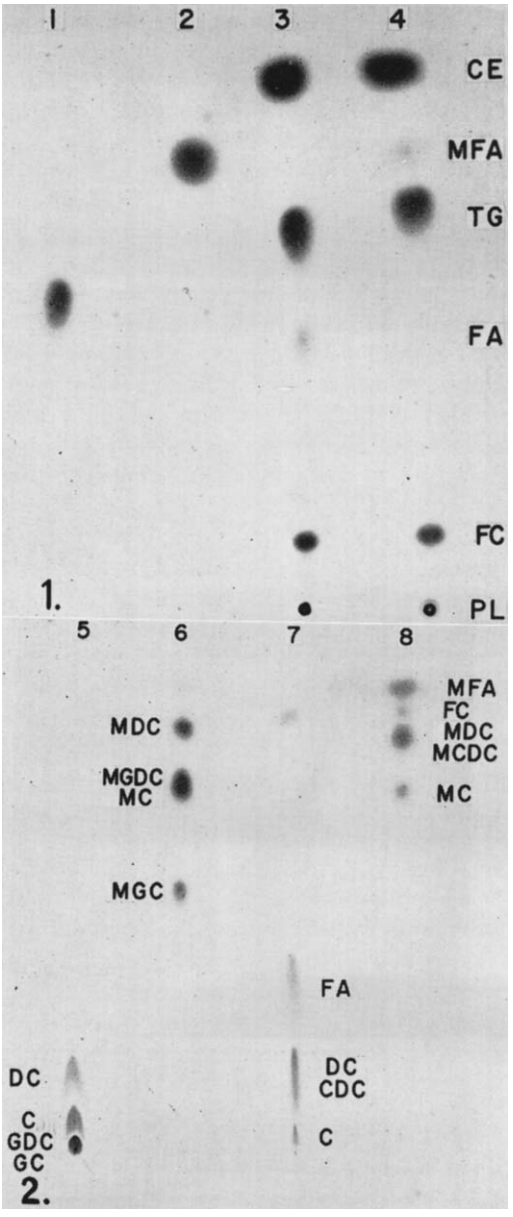

FIG. 1. Thin-layer chromatogram showing: (1) Mixture of fatty acids (FA) containing palmitic, oleic and linoleic acids (10 μg). (2) Same as (1) after treatment with diazomethane to give methyl esters (MFA). (3) Lipid extract of plasma (10 μl) containing cholesterol esters (CE), triglycerides (TG), free fatty acids (FA), free cholesterol (FC) and phospholipids (PL) at the origin. (4) Same as (3) but after methylation, showing quantitative conversion of free fatty acids to their methyl esters (MFA).

FIG. 2. Thin-layer chromatogram showing: (5) Mixture of bile acids containing glycocholic (GC), glycodeoxycholic (GDC), cholic (C) and deoxycholic (DC) acids. (6) Same as (5) but after treatment with diazomethane, showing quantitative conversion of all bile acids to their corresponding methyl esters. (7) Lipids of saponified bile showing cholic acid (C), chenodeoxycholic acid (CDC), deoxycholic acid (DC), free fatty acids (FA) and free cholesterol (FC). (8) Same as (7) but after treatment with diazomethane to give methyl esters of the acids.

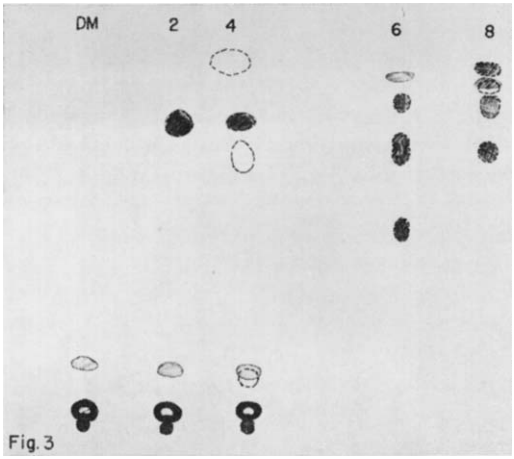

FIG. 3. Diagrammatic representation of autoradiogram of chromatograms. (DM) diazomethane alone; 2 and 4 as in Fig. 1; 6 and 8 as in Fig. 2.

found to be 0.41 meq per liter when calculated from the activity of the standards (Table II).

TABLE I. Activity Present in Methylesters Following Treatment of Acids with C^{14}-Diazomethane.

		Counts per minute	
(a)	Wt stearic acid used, μg	Standard stearic acid alone	Stearic acid added to 20 μl of acid-free plasma lipids
	20	40	39
	40	82	84

(b) Wt cholic acid used, μg	Counts per minute	
150	286	
200	393	

TABLE II. Activity Present in Methylesters from Lipids Following Treatment with C^{14}-Diazomethane.

Lipid treated	Counts/min
1. Total lipids from 20 μl human plasma	135
2. As 1, but free fatty acids removed	9
3. As 2, but 5 μg stearic acid added	295
4. 5 μg stearic acid alone	287

Discussion. It appears that complete methylation of FFA and bile acids (free or conjugated) can be achieved with diazomethane under conditions in which cholesterol, triglycerides and cholesterol esters appear to remain unaltered, as suggested by their mobility on thin-layer chromatography. Other components of plasma or bile lipid extracts may react with diazomethane under the conditions used, including bilirubin* and certain phospholipids(6). However, our results for plasma FFA suggest that there is no interference from such reaction products, or from the substances derived from diazomethane itself, for example, polymethylenes(7). Possible interference by phospholipids in the determination of bile acids in plasma or bile may be avoided by saponification, which removes phospholipids and gives a simplified mixture of non-conjugated bile acids (Fig. 2).

A recent report(8) describing the measurement of standard bile acids by methylation with C^{14}-diazomethane followed by autoradiography of paper chromatograms lends support to the procedure described above.

Summary. As an approach to the problem of microdetermination of acidic substances of biological interest, the procedure of introducing a C^{14}-methyl group and measuring activity associated with the methyl esters appears to offer promise. Treatment with C^{14}-diazomethane under mild conditions results in complete methylation of non-esterified fatty acids in plasma and of bile acids in bile. C^{14}-methyl esters of specific bile acids may be isolated by thin-layer chromatography free from other active substances. Preliminary studies suggest that absolute amounts of free acids present in plasma may be determined by the use of C^{14}-labeled standards.

* Unpublished observations.

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Nucleic Acid Content of Rat Mammary Gland After Teat Ligation.* (28471)

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Selye(1), using histological methods, reported that the suckling stimulus during lactation prevented involution of galactophore-ligated glands of rats up to 14 days. The effects of accumulated milk, however, eventually resulted in mammary involution despite continued suckling(2). Deoxyribonucleic acid (DNA) content of teat-ligated glands of lactating mice was similar to that of intact glands of normal mice whereas contralateral, non-ligated glands contained more DNA than intact glands(3). In guinea pigs suckling did not prevent involution of teat-ligated glands(4,5), and in mice ribonucleic acid (RNA) content and RNA/DNA ratio were very low in teat-ligated glands in comparison with contralateral, non-ligated glands.

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