9. Roosa, R. A., Wilson, D., Defendi, V., Fed. Proc., 1963, v22, 599.

10. Sherman, J. D., Dameshek, W., Nature, 1963, v197, 649.

11. Defendi, V., Roosa, R. A., Koprowski, H., Thymus in Immunobiology, R. A. Good, A. E. Gabrielsen, Ed., Hoeber-Harper, New York, 1964, p504.

12. Defendi, V., Roosa, R. A., The Thymus, Wistar Inst. Symposium Monograph No. 2, 1964, (V. Defendi, D. Metcalf, Ed.), Wistar Inst. Press, Philadelphia, p121.

13. ———, Fed. Proc., 1964, v23, 393.

14. Billingham, R. E., Transplantation of Tissues and Cells, R. E. Billingham, W. K. Silvers, Ed., Wistar Inst. Press, Philadelphia, 1961, p1.

15. East, J., Parrott, D. M. V., Chesterman, F. C., J. Exp. Med., 1963, v118, 1069.

16. Azar, H. A., Williams, J., Takatsuki, K., The Thymus, Wistar Inst. Symposium No. 2, 1964, (V. Defendi & D. Metcalf, Ed.), Wistar Inst. Press, Philadelphia, p75.

17. Wilson, R., Sjodin, K., Bealmar, M., ibid., p89.

18. Miller, J. F. A. P., Science, 1964, v144, 1544.

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A Method for Determination of Plasma Free Fatty Acids.* (29912)

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It has been shown that carboxylic acids may be quantitatively converted to their C^{14}-methyl esters by treatment under mild conditions with an ethereal solution of C^{14}-diazomethane(1). We present here a simpler method for isolation and quantitation of C^{14}-methyl esters derived from plasma FFA.

Methods and results. Lipids were extracted from plasma and washed using the method of Folch *et al*(2); water containing 0.04% (w/v) calcium chloride was used as wash solution to prevent loss of FFA at this stage (3). Six ml of an ethereal solution of C^{14}-diazomethane was prepared from 1 g of N-methyl-N-nitroso-*p*-toluene sulfonamide (New England Nuclear Corp.) containing about 25 μc of radioactivity. The solution of C^{14}-diazomethane was stored in a closed container in a freezer and used within 2 days of preparation; transfer to reaction tubes was made with a pre-chilled pipette to minimize evolution of gas. All operations with diazomethane were conducted in a fume hood. Columns of silicic acid (Bio-Rad 325 mesh, activated at 110° for 3 hours) were prepared and washed thoroughly with petroleum ether, b.p. 30-60° (PE) in glass tubes (200 × 8 mm).

For estimation of plasma FFA, the lipid extracts from plasma (0.1-0.5 ml) were evaporated to dryness under nitrogen in a 10 ml glass-stoppered centrifuge tube, and immediately redissolved in one drop of methanol. Ten drops of the fresh ethereal solution of C^{14}-diazomethane were added, the tubes stoppered, mixed and placed in the dark at room temperature for 30 minutes, with swirling after 15 minutes. The solvents and excess diazomethane were evaporated under nitrogen, and the residues transferred quantitatively with a total of 12 ml of PE to 3-cm columns of silicic acid. The PE emerging from the columns was discarded, and elution with 20 ml of 2% (v/v) diethyl ether in PE was commenced at once. The whole of the eluate was collected in 20 ml counting vials, the solvent evaporated and the residue dissolved in scintillation fluid and counted to give a standard deviation of less than 2%.

Known amounts (usually 25-250 μg) of pure palmitic or stearic acid were treated as described above and from the results a standard curve of radioactivity *vs* weight of FFA was constructed.

A blank determination was also carried through, in which the C^{14}-diazomethane was added to a tube containing one drop of methanol only. The count from this tube

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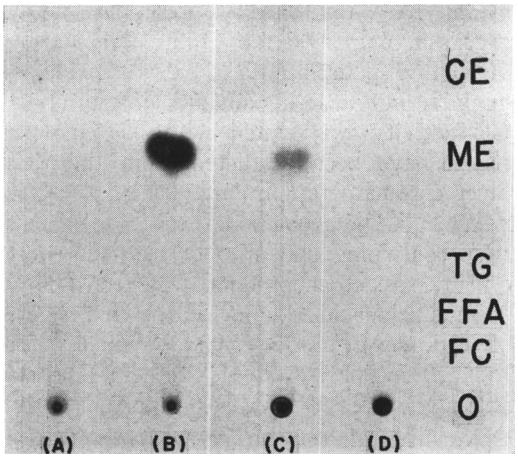

FIG. 1. Autoradiogram of thin-layer chromatogram showing absence of significant radioactivity in the methyl ester region when free fatty acids are absent from the material methylated with C^{14}-diazomethane. (a) No addition; (b) palmitic and oleic acid mixture; (c) total plasma lipids; (d) plasma lipids with free fatty acids removed. CE, region on chromatogram occupied by cholesterol esters; ME, methyl esters; TG, triglycerides; FFA, free fatty acids; FC, free cholesterol; O, phospholipids at origin.

was always virtually background.

Distribution of radioactivity: autoradiography. It is important in this procedure that radioactivity in any form other than methyl esters should be well clear of the methyl ester region during chromatography, and that plasma lipids other than FFA do not yield radioactive methyl esters. This was checked by thin-layer chromatography and autoradiography, as follows: Ethereal C^{14}-diazomethane (200 μl) was added to each of 4 glass-stoppered centrifuge tubes containing one drop of methanol and, respectively, (a) no other addition; (b) 20 μg each of palmitic and oleic acids; (c) lipid extract of 20 μl normal plasma (FFA content about 3 μg); and (d) lipid extract from 20 μl of normal plasma from which FFA had been removed by recent passage through a basic ion-exchange resin(1). The tubes were stoppered, mixed and left in the dark for 30 minutes, after which solvents and excess diazomethane were removed under nitrogen. The contents of the tubes were transferred in chloroform to thin-layer plates coated with Silica Gel G (E. Merck A. G.) as single spots, and the plates developed with a mixture of PE-diethyl-ether-glacial acetic acid 92/8/1 (v/v). An autoradiogram made from the developed plate (4-week exposure) is shown in Fig. 1. All samples showed radioactivity at the origin, and there was evidence of additional radioactive components in this region with the plasma samples. Minor amounts of radioactivity were present between the point of application and the free cholesterol region. Methyl ester radioactivity was seen only where FFA were present in the sample.

After autoradiography, the thin-layer plate was visualized by spraying with 50% (v/v) sulfuric acid and heating at 250°. Visual inspection of the chromatogram showed that complete conversion of FFA to methyl esters had occurred, and confirmed that methyl esters were not formed from any component of the FFA-free plasma.

Distribution of radioactivity: liquid scintillation counting. Pure stearic acid (10-30 μg) or lipid extracts of plasma (50-200 μl) were treated with C^{14}-diazomethane and chromatographed on thin-layer plates, as described in the previous section. Following development, the silicic acid was scraped from appropriate regions of the plate with a razor blade, and the lipids eluted from small glass columns directly into counting vials. After evaporation of the solvents and addition of scintillation fluid the counts present in various areas were recorded. The results of two such experiments are shown in Fig. 2.

There was considerable radioactivity at the origin, the amount being greater with plasma than with pure stearic acid. There was significant activity in the free cholesterol region and immediately in front of it. A clear peak of radioactivity was observed in the methyl ester area, with only very minor amounts of activity on each side of it. It was observed that with increase of plasma volume (amount of C^{14}-diazomethane used being kept constant), the methyl ester radioactivity peak increased without increase of radioactivity in the adjacent chromatographic areas.

Separation of methyl ester radioactivity on silicic acid columns. Experience with thin-layer chromatography (Fig. 2) suggested that complete elution of the cholesterol ester and methyl ester regions of methylated plas-

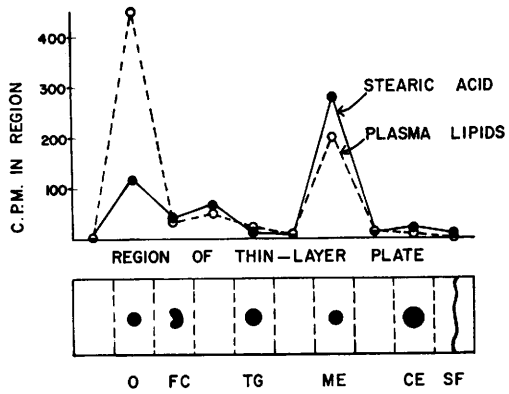

FIG. 2. Distribution of C^{14}-radioactivity on thin-layer chromatograms following methylation of stearic acid (10 μg) and of plasma lipids (50 μl plasma) with C^{14}-diazomethane. SF, solvent front; other legend, as Fig. 1.

ma lipids, using small silicic acid columns, would provide a simple method for isolating methyl ester activity from other radioactivity present in the total sample. Preliminary experiments showed that use of 3-cm columns of silicic acid (8 mm diameter) provided rapid separation of these lipids, when eluted with 20 ml of 2% (v/v) diethyl ether in PE. Triglycerides and more polar lipids remained on the columns.

The procedure was tested as follows: 9,10-T-palmitic acid was first purified by thin-layer chromatography and a known amount of tritium (associated with a negligible amount of palmitic acid) was added to a plasma lipid extract as a label. The mixture was then methylated with C^{14}-diazomethane as described, and the reaction products transferred quantitatively to a 3-cm silicic acid column with 12 ml of PE. The PE eluate was collected in a counting vial. Elution with 2% (v/v) diethyl ether in PE was then commenced, and 7 consecutive 5 ml fractions collected in counting vials. Finally the column was washed with 20 ml of methanol to give fraction 9. The solvents were evaporated and the residues counted for C^{14} and tritium, with the results shown in Table I. Radioactivity eluted by 12 ml of PE was negligible. Overall recovery of tritium radioactivity, added originally as palmitic acid, was better than 97%, and a satisfactory proportion of this was recovered in the first 20 ml of a 2% diethyl ether-PE eluate (94.2% overall). C^{14} radioactivity present in methyl groups accompanied the tritium radioactivity of methyl palmitate in a very satisfactory manner, but a large amount of more-polar C^{14} activity was eluted with methanol, as might have been predicted from the thin-layer chromatographic data.

When C^{14}-diazomethane was added to a tube containing one drop of methanol only, and the reaction product transferred to a 3-cm silicic acid column and eluted as described, the C^{14} radioactivity obtained in the first 20 ml of 2% diethyl ether-PE eluate was always essentially background.

Replicate determinations of plasma FFA. From a washed Folch extract of plasma, 9 identical aliquots were taken, each containing lipids from 0.5 ml plasma. These were treated with C^{14}-diazomethane as described, and FFA content determined, using stearic acid standards. The results obtained from a typical experiment are shown in Table II. The standards gave a good straight line passing through the origin. The replicates gave FFA values from 74-80 μg per 0.5 ml plasma, with a mean of 76.8 μg, or 0.541 $\pm$ 0.011 meq per liter (standard deviation). Per cent standard error for 9 determinations was $\pm$ 0.67.

Comparison with other methods. The FFA content of 4 plasma samples each from 2 subjects (W and S) was determined in duplicate by the C^{14}-diazomethane procedure (0.5 ml plasma) and also by 2 other methods: the Michaels method(4); and measurement of FFA spot size on thin-layer chromatograms of plasma extracts(5). The samples were ob-

TABLE I. C^{14}- and Tritium Radioactivity Eluted by Solvents from 3-Centimeter Column of Silicic Acid.

Fraction No.	Solvent & vol	Tritium (c.p.m.)	C^{14} (c.p.m.)
1	12 ml PE*	17	0
2	5 ml 2% DE†	17	0
3	" "	13733	14446
4	" "	248	245
5	" "	122	107
6	" "	86	90
7	" "	79	58
8	" "	59	35
9	20 ml methanol	238	18280

* Petroleum ether, b.p. 30-60°.
† 2% (v/v) diethyl ether in petroleum ether.

TABLE II. C^{14}-Radioactivity in Methyl Esters from Stearic Acid Standards and from Free Fatty Acids of Plasma.

	μg taken	c.p.m. recorded
Stearic acid standards:	50	855
	100	1611
	150	2367
	200	3325
	250	4163
	c.p.m. recorded	μg FFA calculated from standard curve
Replicate samples of plasma lipids:	1265	76
	1257	76
	1276	77
	1261	76
	1345	80
	1276	77
	1232	74
	1299	78
	1281	77

tained (a) following a 16-hour fast; (b) immediately after vigorous exercise for 5 minutes (FFA level falls); (c) after a further 1-hour period of exercise (FFA level rises above fasting); and (d) after a further period of 30 minutes rest (FFA level rises still further). The results are given in Table III.

Discussion. Work previously reported(1) and the present results using thin-layer chromatography have demonstrated that virtually quantitative conversion of FFA, saturated or unsaturated, to methyl esters may be obtained by treatment with diazomethane. It has now been further established that fatty acid methyl ester radioactivity is produced during the interaction of C^{14}-diazomethane and plasma lipids only when FFA are present; and that significant radioactivity is not present in the regions adjacent to that of fatty acid methyl esters during thin-layer chromatography.

Sharp separation of radioactivity due to C^{14}-methyl esters of FFA from other radioactivity in the reaction mixture was conveniently achieved by elution from 3-cm columns of silicic acid with 20 ml of 2% (v/v) diethyl ether in PE. The eluate contained the cholesterol esters and a satisfactory proportion of the total methyl esters added to the column. The satisfactory manner in which C^{14}-radioactivity from plasma FFA methyl esters accompanied tritium radioactivity due to methyl palmitate during elution of the column indicated that the methyl esters of the different FFA components of plasma (oleic, palmitic, stearic, linoleic acids, and others) are eluted quite sharply and without undue trailing in this system.

Further elution with methanol removed from the column a considerable amount of C^{14}-radioactivity derived from diazomethane, but not accompanied by significant tritium activity. Autoradiography (Fig. 1) showed that this polar radioactivity was of two types: one derived from diazomethane alone, and possibly due to polymethylenes; and the second probably due to interaction of certain plasma components (bilirubin, bile acids, some phospholipids) with diazomethane(1).

0.1 ml of plasma or less may be used for a single determination, particularly if the specific activity of the C^{14}-diazomethane is increased, or the counting time lengthened. Any losses of radioactivity from the stock ethereal C^{14}-diazomethane solution, by evaporation, should not change the specific activity of the reagent, upon which the count produced by a fixed weight of FFA depends; but standards must be run with each new preparation of diazomethane.

Summary. A method for determination of free fatty acids in plasma is presented, together with tests for the validity of the procedures used. Total lipids are treated with excess ethereal C^{14}-diazomethane, and radioactivity due to C^{14}-methyl esters of fatty acids is isolated by silicic acid column chromatography, and counted. Similar treatment of known amounts of standard fatty acid

TABLE III. Free Fatty Acid Values* Found for 8 Plasma Samples by 3 Methods.

Sample No.	Free fatty acids (meq per liter) Michaels method	Spot size method	Diazomethane method
W (a)	.78	.79	.65
W (b)	.64	.62	.60
W (c)	1.24	1.14	1.26
W (d)	1.52	1.51	1.48
S (a)	1.06	1.19	1.07
S (b)	.57	.55	.56
S (c)	1.84	1.83	1.70
S (d)	1.95	2.01	1.77

* Mean of 2 determinations in each case.

yields a curve from which the plasma free fatty acid concentration may be obtained. Use of 0.5 ml of plasma per determination gives optimal results, but the method may be employed with 0.1 ml plasma or less.

1. Sodhi, H. S., Wood, P. D. S., Proc. Soc. Exp. Biol. and Med., 1963, v113, 714.

2. Folch, J., Lees, M., Sloane-Stanley, G. H., J. Biol. Chem., 1957, v226, 497.

3. Entenman, C., J. Am. Oil Chem. Soc., 1961, v38, 534.

4. Michaels, G., Metabolism, 1962, v11, 833.

5. Schlierf, G., Wood, P. D. S., J. Lipid Res., in press.

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Modification of Sucrose Adaptation in the Rat. (29913)

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Rats injected intraperitoneally (IP) with hypertonic sucrose solutions adapt to subsequent reexposures to the disaccharide by increasing 1) volume output of urine, 2) sucrose concentration of urine and 3) fraction of the injected load excreted within a 6-hour test period(1). Similar experiments employing intraarterial (IA) loading of the sugar demonstrated that the adaptation generated under these circumstances is qualitatively different from that induced by IP administration. Under conditions of IA infusion, reexposure to sucrose causes a reduction in both total urine volume and the fraction of the load excreted in 6 hours, with no demonstrable difference in sucrose concentrations of urine(2). To determine whether the reductions in urine volume and sucrose excretion after a second IA infusion persist beyond the 6-hour period, experiments were conducted to examine the completeness of sucrose excretion over 24 hours. In addition a series of repeated IA loadings was performed to determine if the adaptive response, once established, was further modified.

Methods. Three groups of male albino rats (Holtzman, 188-239 g) were infused with 2.0 ml/100 g body weight (b.w.) of a 1.46 M solution of sucrose through a chronically indwelling catheter. The tip of the catheter rested in the aortic arch. Prior experiments demonstrated that this load given IA produced a maximum adaptive response with respect to total urine and sucrose excreted. At the time of loading animals were unanesthetized and under moderate restraint(3). All urine voided within the 24-hour period following infusion was collected and analyzed for sucrose(4). Infusions of the same relative load were repeated in each group at 6-day intervals and totaled 4 exposures per group. Urine volumes excreted and urinary sucrose concentrations were measured following each exposure. Data were treated by analysis of variances of independent group means.

Results. A comparison of the sucrose excretion after 6 and 24 hours in unadapted and adapted rats is given in Table I. Unadapted rats (first exposure) excreted most of the load (92.8%) within the 6-hour period following the infusion and increased the fraction of the total load excreted in 24 hours by merely 0.7%. In contrast, adapted animals (second exposure) excreted only 78.5 ± 4.1% (S.D.) of the infused load within

TABLE I. Recovery of Sucrose from Urine as Percentage of Infused Load.*

	Time from infusion		Δ
Exposure	6 hr	24 hr	6-24 hr
1	92.8 ± 6.2† (13)‡	93.5 ± 4.7 (33)	.7
2	78.5 ± 4.1 (12)	85.9 ± 4.2 (30)	7.4
ΔExposure 1-2	14.3	7.6	

* 2.0 ml/100 g body weight of 1.46 M sucrose (50%); intraarterially.
† Mean ± S.D.
‡ No. of animals infused.