Biosynthesis of Fatty Acids in Obese Mice *in Vivo*. II. Studies with DL-Malate-2-^{3}H-3-^{14}C, Succinate-2,3-^{3}H-2,3-^{14}C, and DL-Isocitrate-2-^{3}H-5,6-^{14}C*

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ABSTRACT: Biosynthesis of fatty acids in the liver and in other tissues of the remaining carcass of obese hyperglycemic mice and their lean siblings has been investigated by isolation and counting of radioactivity in total fatty acids of mice sacrificed 90 min after intraperitoneal injection of trace amounts of malate, succinate, or isocitrate specifically labeled with tritium and carbon-14. The patterns of relative extent of transfer to liver and carcass fatty acids of ^{3}H and ^{14}C were similar for malate and succinate, and transfer for both compounds was of the same order as earlier observed with pair-labeled lactate and glycerol. The findings are compatible with a coupling of oxidation of all of these substrates with L-malate:oxidized nicotinamide–adenine dinucleotide phosphate oxidoreductase (malic enzyme) followed by subsequent transfer of tritium from reduced nicotinamide–adenine dinucleotide phosphate to fatty acids. This coupling appears to have particularly high activity in the liver. Theoretical considerations of different intracellular sites of metabolism of malate and succinate suggest a compartmental advantage of mitochondrially formed malate over exogenous malate in the transfer of metabolic reducing hydrogen. In contrast to findings with DL-malate-2-^{3}H and succinate-2,3-^{3}H transfer of ^{3}H from DL-isocitrate-2-^{3}H was more extensive for the carcass than for the liver fatty acids, as was found previously with glucose-1-^{3}H. A significant contribution of reducing equivalents *via* D-*threo*-isocitrate:oxidized nicotinamide–adenine dinucleotide phosphate oxidoreductase in some nonhepatic tissues is indicated. On the other hand, the conversion of ^{14}C from DL-isocitrate-5,6-^{14}C (*via* aconitase and citrate lyase) was more extensive in liver than in other tissues. All ^{3}H- and ^{14}C-labeled carbohydrates in the present study, like those previously tested, were converted into hepatic fatty acids of obese mice in severalfold higher extent than to those of lean mice, whereas conversion into total fatty acids of the carcass was only moderately higher in the obese. However, the range of differences among labeled carbohydrates for labeling of fatty acids of the liver of obese *vs.* lean mice was greater than previously found. Thus, the obese:lean ratio was only 3-fold for DL-malate-2-^{3}H but as high as 12-fold in the case of succinate-2,3-^{14}C. Unlike findings with labeled carbohydrates related to the glycolytic sequence, the formation of $^{14}CO_2$ from ^{14}C-labeled malate, succinate, and isocitrate was not lower in obese mice than in lean mice.

A previous publication from this laboratory (Shreeve *et al.*, 1967) reported that tritium transferred during the oxidation of labeled lactate and glycerol *in vivo* is utilized for reduction of intermediates formed during biosynthesis of fatty acids. As found with the obese hyperglycemic mouse and its lean siblings, this was particularly true for the liver and to a lesser extent for the other tissues included collectively in the headless carcass. Comparisons of isotopic yields from DL-lactate-2-^{3}H, glycerol-2-^{3}H, glucose-1-^{3}H, and glucose-6-^{3}H and from corresponding ^{14}C-labeled carbohydrates suggested that in the liver transfer of protons from the three-carbon precursors is more "efficient" than that from glucose. These studies were consistent with earlier reports showing biosynthesis of fatty acids from DL-lactate-2-^{3}H (Lowenstein, 1961a; Foster and Bloom, 1961) and glycerol-2-^{3}H (Foster and Bloom, 1961) in rat liver slices, from L-lactate-2-^{3}H in perfused rat liver (D'Adamo *et al.*, 1961), and from DL-lactate-2-^{3}H in human subjects *in vivo* (Ghose *et al.*, 1964). The above-cited evidence for major substrate sources of reducing hydrogen other than glucose is in accord with calculations that under certain conditions the pentose cycle can provide only one-half to three-fourths of the reducing equivalents necessary for biosynthesis of fatty acids in adipose tissue *in vitro* (Flatt and Ball, 1964; Katz *et al.*, 1966).

Marked preferences of the various enzymes comprising the "fatty acid synthase" complex for the reducing coenzyme, NADPH, rather than NADH has been clearly shown *in vitro* (Gibson *et al.*, 1958). Assuming that this finding is a reflection of *in vivo* specificities, significant contribution of reducing hydrogen from NAD^{+}-linked substrates, such as lactate and glycerol, must be somehow reconciled with the preference for NADPH demonstrated *in vitro*. Of interest, therefore, are the reports (Young *et al.*, 1964; Pande *et al.*, 1964; Leveille and Hanson, 1966) of parallel changes in the activity of "malic enzyme" (L-malate:NADP^{+} oxidoreductase (decarboxylating), EC 1.1.1.40) and fatty acid synthesis in liver and adipose tissue. Such measurements support suggestions (Lowenstein, 1961a; Young *et al.*, 1964; Pande *et al.*, 1964; Ball, 1966; Shreeve *et al.*, 1967) of a possible key role of malate as a mediator of proton transfer to fatty acids *via* a coupling of various NAD^{+}-linked reactions through NAD^{+}-dependent L-malate dehydrogenase (EC 1.1.1.37) to NADP^{+}-dependent "malic enzyme."

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Substrates other than those mentioned above have also been suggested as possible contributors *via* pyridine nucleotides of reducing equivalents in the biosynthesis of fatty acids. Thus, isocitrate *via* its cytoplasmic dehydrogenase (D-*threo*-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42) could conceivably transfer protons directly to $NADP^+$ and thence to fatty acids (Lowenstein, 1961b). Also, tritium from succinate-2,3-^3H added *in vitro* to mitochondria of rat aorta has been identified in fatty acids (Whereat, 1965). The relevance of this finding to fatty acid synthesis *in vivo* is uncertain. Furthermore, the relationship of the oxidation of succinate to the generation of reduced NAD^+ remains controversial, particularly in its quantitative aspects (Gawron *et al.*, 1964; Hoberman *et al.*, 1964; Chance *et al.*, 1965; Griffiths and MacNeice, 1965; Krebs, 1967).

In order to evaluate further the potential of these carbohydrates to donate protons for fatty acid synthesis *in vivo*, the studies reported herein extend our previous observations to results obtained with labeled malate, succinate, and isocitrate.

Materials and Methods

Succinic acid-2,3-^3H (specific activity of 130–139 mCi/mmole), DL-malic acid-2-^3H (65 mCi/mmole), and DL-isocitric acid-2-^3H and -5,6-^{14}C, containing approximately 50% DL-*allo*-isocitric acid by company assay (70 and 2.8 mCi per mmole, respectively), were obtained from New England Nuclear Corp., Boston, Mass. Succinic acid-2,3-^{14}C (5.7 mCi/mmole) and DL-malic acid-3-^{14}C (12 mCi/mmole) were obtained from Volk Radiochemical Co., Chicago, Ill. Labeled compounds were diluted in sterile 0.9% NaCl.[1] Amounts given to each obese or pair of lean mice were as follows: succinate, 40–300 mCi of ^3H and 5–10 μCi of ^{14}C; malate, 40–50 μCi of ^3H and 4–5 μCi of ^{14}C; isocitrate, 50 μCi of ^3H and 5 μCi of ^{14}C. No differences were found over the large range of succinate used.

As in the preceding study (Shreeve *et al.*, 1967) the animals used were the obese hyperglycemic mice of the strain, C57BL/6J obob, from the Jackson Laboratory, Bar Harbor, Me., and their lean siblings. Two lean mice were paired for comparison against a single obese litter mate. Obese mice ranged in weight from 37 to 64 g (mean = 49.2 g), and the range in weight per pair of lean mice was 38–63 g (mean = 46.6 g). Mice ranging in age from 8 to 24 weeks were used with approximately equal distribution between male and female animals. Weights of livers from obese mice ranged from 2.1 to 4.7 g (mean = 3.14 g) and those of paired livers from lean animals ranged from 1.4 to 2.5 g (mean = 1.90 g). Thus, the ratio of liver weight: body weight was approximately 50% greater in the obese mice than in the lean. Groups of animals studied with each compound were comparable with respect to distribution of age, sex, and total body and liver weights. The range of blood glucose values was as reported earlier.

The experimental protocol was essentially as described previously (Shreeve *et al.*, 1967). Mice were fasted for 5 hr before intraperitoneal injection of a dilution of labeled carbohydrate (either ^3H or ^{14}C or both). No carrier was added to any of the original labeled materials. One milliliter, containing the radioactivity noted above, was administered to each obese mouse and 0.5 ml to each lean sibling. Each obese mouse or pair of lean mice was then immediately placed in a separate metabolism cage which enabled collection of $^{14}CO_2$ in the expired air for 90 min. Techniques of sacrifice and subsequent handling of blood and tissues as well as methods for saponification and extraction of fatty acids, analysis of ^3HOH and glucose in blood and of $^{14}CO_2$ in expired air, assay of radioactivity, and calculation of the conversion of ^3H from labeled precursors into ^3HOH were as described in earlier publications from this laboratory (Shigeta and Shreeve, 1964; Shreeve *et al.*, 1967).

Results

Conversion of Labeled Carbohydrates into Total Fatty Acids of the Liver. PER CENT DOSE. Table I contains values for percentages of total administered ^3H or ^{14}C (malate, succinate, or isocitrate) which were found in the total fatty acids of the liver after saponification. In the case of DL-malate-2-^3H, it may be assumed that only the natural (L) isomer contributes tritium to fatty acids *via* pyridine nucleotides, because D-malic acid dehydrogenase does not utilize either NAD^+ or $NADP^+$ (Tubbs, 1965). This situation is analogous to that for DL-lactate-2-^3H (Shreeve *et al.*, 1967). However, for L-malate-2-^3H, there is the additional consideration that rapid equilibration of malate with fumarate would occur in the liver (Hoberman and D'Adamo, 1962; Haynes, 1965). Since the inner carbon atoms of fumarate are randomized in the rehydration of the molecule, half of the tritium would be shifted to C-3 of L-malate.[2] While a very minor amount of this tritium on C-3 could carry over through acetyl-CoA to fatty acids (Abraham *et al.*, 1963), it would not be available for transfer to pyridine nucleotide. In thus comparing DL-lactate-2-^3H and DL-malate-2-^3H, the latter would appear to contribute *via* pyridine nucleotide a higher percentage of its available tritium to liver fatty acids of lean mice, though less to those of obese mice, than did DL-lactate-2-^3H (in earlier studies[3]). These differences are evident, in another way, as only a 3-fold higher incorporation of DL-malate-2-^3H into liver fatty acids of obese than into those of lean mice (Figure 1) compared with a six-fold difference previously found with DL-lactate-2-^3H. On the other hand, the obese:lean (O:L) ratio for DL-malate-3-^{14}C is 6:1, which approximates that observed for DL-lactate-2-^{14}C as well as for ^3H- or ^{14}C-labeled glucose or glycerol (Shreeve *et al.*, 1967).

The percentage of total dose of succinate-2,3-^3H incor-

[1] All labeled compounds were at least 98% radiochemically pure according to assay with paper or thin-layer chromatography by the commercial vendors. Tests in our laboratory have shown that <1% of tritium is distillable from aqueous solutions of DL-malic acid-2-^3H or succinic acid-2-^3H during early periods of use. New England Nuclear Corp. confirms this finding with freshly dissolved, neutral solutions of sodium DL-malate-2-^3H. However, after months or years of storage in frozen solution with intermittent thawing and use, some samples of DL-malic acid-2-^3H show in our laboratory 10 to 95% distillable tritium, whether from acid or neutral solution. Experimental data in this paper are derived from samples of DL-malic acid-2-^3H which at the time of use contained either <1% or no more than 10% distillable tritium.

[2] The tritium would not be incorporated into water *via* the repeated operation of the fumarase reaction because of the stereospecificity with respect to the hydrogens removed (Hoberman and D'Adamo, 1960).

[3] The values earlier obtained (Shreeve *et al.*, 1967) for mean per cent dose of total DL-lactate-2-^3H in liver fatty acids of lean and obese mice, respectively, were 0.303 and 1.825.

TABLE I: Comparative Transfers of Tritium and Carbon-14 from Various Carbohydrates into Fatty Acids of Liver and Carcass of Lean (L) and Obese (O) Mice.

	DL-Malate[a]				Succinate				DL-Isocitrate			
	-2-^{3}H[b]		-2-^{14}C		-2,3-^{3}H		-2,3-^{14}C		-2-^{3}H		-5,6-^{14}C	
	L[c]	O	L	O	L	O	L	O	L	O	L	O
					Liver							
% dose	0.272	0.824	0.041	0.247	0.263	1.676	0.037	0.439	0.061	0.232	0.010	0.064
(No. of expt)	(7)	(7)	(7)	(8)	(7)	(9)	(6)	(7)	(8)	(8)	(6)	(7)
±SE[d]	0.036	0.045	0.006	0.035	0.024	0.174	0.009	0.081	0.007	0.034	0.002	0.005
p	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	
					Carcass							
% dose	0.244	0.390	1.111	1.772	0.271	0.603	0.926	2.724	0.319	0.353	0.086	0.116
(No. of expt)	(7)	(6)	(8)	(8)	(8)	(11)	(7)	(10)	(7)	(7)	(6)	(7)
± SE[d]	0.032	0.061	0.117	0.326	0.038	0.088	0.151	0.262	0.033	0.032	0.011	0.014
p	<0.005		<0.10		<0.005		<0.001		<0.50		<0.20	

[a] This row across refers to the carbohydrate used. [b] This row across refers to the label used. [c] This row across refers to the type of mouse. [d] $SE = \sqrt{\Sigma(x - \bar{x})^2/[n(n-1)]}$.

porated into liver fatty acids of lean mice is about the same as for DL-malate-2-^{3}H, but is higher for fatty acids of obese mice (Table I). Thus, the O:L ratio for succinate-2,3-^{3}H is about twice as high as for DL-malate-2-^{3}H (Figure 1). With the general assumption that the isotope effect is negligible, the first dehydrogenation of succinate to fumarate would remove half of the tritium through an FAD-coupled reaction. An intramitochondrial system provides for the possible subsequent transfer of protons from $FADH_2$ to NAD^+ by an energy-linked reversal of phosphorylation (Chance *et al.*, 1965). According to some evidence with mitochondria of aorta (Whereat, 1965) and heart (Griffiths and MacNeice, 1965) tritium could be conveyed to fatty acids in this process. However, other observations with labeled succinate (Hoberman *et al.*, 1964) and the predictability of loss to water from the β locus of NADH in flavoprotein-catalyzed reactions (Hoberman *et al.*, 1964; Griffiths and MacNeice, 1965) would suggest that little, if any, tritium in fatty acids derives in this way. If the above considerations apply *in vivo*, the values for per cent total dose incorporated from DL-malate-2-^{3}H and succinate-2,3-^{3}H (Table I) may be directly compared with each other, since the remaining 50% of total tritium on fumarate and L-malate formed from succinate corresponds in general to the 50% of DL-malate which is available as L-malate. Furthermore, since only half of the tritium of L-malate is available for transfer to pyridine nucleotides, the values for both succinate and malate in Table I should theoretically be multiplied by four to approximate the true radiochemical yield.

As in the case of the ^{3}H-labeled analogs, about the same amount of DL-malate-3-^{14}C and succinate-2,3-^{14}C are transferred to fatty acids of lean mice, but more succinate-2,3-^{14}C is found in the fatty acids of obese mice. The O:L ratio of 12:1 for liver fatty acids formed from succinate-2,3-^{14}C is higher than for other labeled compounds in the present study and also higher than observed previously with labeled glucose, lactate, or glycerol (Shreeve *et al.*, 1967).

Evaluation of per cent dose incorporated into fatty acids from DL-isocitrate-2-^{3}H-5,6-^{14}C requires an additional consideration of isomeric specificity. The values as given in Table I are those for the total DL-*threo* and DL-*allo* mixtures. DL-*allo*-Isocitrate has been shown to be enzymatically inert.[4] Little is known about the metabolism of L-*threo*-isocitrate; perhaps of the four isomers only the natural D-*threo*-isocitrate would contribute tritium in pyridine nucleotide catalyzed reactions. Consideration of the amount of $^{14}CO_2$ and ^{3}HOH formed (see below and Table II) would suggest that none of the DL-*allo* but at least some of the L-*threo* isomer is metabolized to these catabolic products, if not to reducing hydrogen.

The percentage of tritium transferred to liver fatty acids from isocitrate-2-^{3}H, even after accounting for the relative specificities, still appears minor compared with L-malate or with L-malate formed from succinate. The O:L ratio (*ca.* 4:1) is in the same range as for DL-malate-2-^{3}H. Nevertheless, as for all other labeled compounds, there is a marked difference between lean and obese in the extent of incorporation.

In the case of DL-isocitrate-5,6-^{14}C, the tertiary carboxyl (C-6) would be converted either into CO_2 or into carboxyl groups of C_4 acids and thus would not contribute to ^{14}C in fatty acids. Position C-5 of isocitrate becomes that carbon of citrate which, when acted upon by citrate lyase (EC 4.1.3.6), converts into the carboxyl carbon of acetyl-CoA (Spencer and Lowenstein, 1962) and thereby may be converted into fatty acids; *via* the forward operation of the Krebs cycle, *i.e.*, successive formation of oxalosuccinate, 2-ketoglutarate, and C_4 dicarboxylic acids, C-5 of isocitrate would be converted into carboxyl carbons of C_4 acids. There it would be either unavailable for formation of fatty acids or at most far less so than *via* the citrate cleavage reaction (Spencer and Lowen-

[4] Assay of New England Nuclear Corp.

TABLE II: Conversions of Tritium and Carbon-14 from Various Carbohydrates into Body Water and Respiratory Carbon Dioxide of Lean (L) and Obese (O) Mice.

	DL-Malate[a]				Succinate				DL-Isocitrate			
	-2-^{3}H[b]		-3-^{14}C		-2,3-^{3}H		-2,3-^{14}C		-2-^{3}H		-5,6-^{14}C	
	L[c]	O	L	O	L	O	L	O	L	O	L	O
% dose in body water or respiratory CO_2	"112"	"132"	50	47	83	89	67	63	33	41	25	25
(No. of expt)	(7)	(7)	(8)	(8)	(8)	(10)	(5)	(6)	(7)	(7)	(5)	(5)
±SE[d]	4.3	5.4	1.4	3.3	6.5	8.7	1.7	4.0	1.5	2.3	0.7	1.1
p	<0.02		<0.40		<0.70		<0.50		<0.02		<0.60	

[a] This row across refers to the carbohydrate used. [b] This row across refers to the label used. [c] This row across refers to the type of mouse. [d] SE = $\sqrt{\Sigma(x - \bar{x})^2/[n(n-1)]}$.

stein, 1962). The amount of ^{14}C converted into fatty acids from DL-isocitrate-5,6-^{14}C suggests that the active isomer of this compound is converted to citrate and then to fatty acids in the liver to an extent somewhat like that for ^{14}C-labeled malate and succinate. The O:L ratio of 6:1 is that which is commonly found for other labeled compounds (Figure 1) (Shreeve *et al.*, 1967).

^{3}H:^{14}C RATIO. A comparison of the amount of ^{3}H *vs.* the amount of ^{14}C incorporated for any one compound in experiments in which both isotopes were simultaneously administered (Figure 2) provides another measure of the relative "efficiency" of transfer of ^{3}H to fatty acids. To some extent, this approach compensates for differences among compounds in delivery to the metabolic site and in pool dilution prior to metabolism. However, differential dilution after separation of ^{3}H and ^{14}C in the course of metabolism can also cause differences in this ratio among compounds and between different organ sites, *e.g.*, liver and carcass (Volokhine *et al.*, 1968).

The ^{3}H:^{14}C ratios in fatty acids of the liver of lean mice are essentially equal for malate and succinate and of the same order as previously observed for pair-labeled lactate and glycerol (Shreeve *et al.*, 1967). The transfer to liver fatty acids of ^{3}H relative to ^{14}C from malate[5] and succinate appears to be, like that from lactate and glycerol, generally of a higher order than from glucose labeled at either the 1 or 6 positions (Shreeve *et al.*, 1967). For obese mice, the ^{3}H:^{14}C ratios from malate or succinate are lower than for lean mice and in the case of malate significantly so ($p < 0.01$) (Figure 2). This difference was not observed in the case of labeled lactate or glycerol.

The ^{3}H:^{14}C ratio for DL-isocitrate, although approximately the same for liver fatty acids as the ratios for malate and succinate, has little significance in relation to the latter because of the disparate types of labeling with ^{14}C and the different metabolic pathways involved. Of interest, however, may be the lower ^{3}H:^{14}C ratio for obese mice than for lean mice given DL-isocitrate, as in the cases of malate and succinate.

Conversion of Labeled Carbohydrates into Total Fatty Acids of the Carcass. PER CENT DOSE. The amounts of ^{3}H transferred to fatty acids of the carcass from malate and succinate (Table I) were of the same order of magnitude. There was no difference in lean mice while succinate-2,3-^{3}H showed moderately higher values for obese mice. Differences between lean and obese mice were lower and of lesser statistical significance generally than for liver fatty acids (Table I). The amounts of ^{3}H transferred from malate and succinate to carcass fatty acids were higher than for DL-lactate-2-^{3}H or glycerol-2-^{3}H, about the same as for glucose-6-^{3}H, and less than for glucose-1-^{3}H (Shreeve *et al.*, 1967). The O:L ratios for ^{3}H- or ^{14}C-labeled malate or succinate (Figure 1) are in the same general range as those for labeled glucose, lactate, and glycerol. The O:L ratios for ^{3}H- and ^{14}C-labeled succinate are slightly higher than those for any of the other labeled compounds.

DL-Isocitrate-2-^{3}H shows comparatively high amounts of tritium transferred to carcass fatty acids compared with liver fatty acids. In this respect, this compound resembles glucose-1-^{3}H more than it does ^{3}H-labeled malate, succinate, lactate, or glycerol. On the other hand, ^{14}C transferred to carcass fatty acids of lean or obese mice from isocitrate-5,6-^{14}C after consideration of stereospecificities is lower than from any of the other ^{14}C-labeled carbohydrates.

^{3}H:^{14}C RATIO. The ^{3}H:^{14}C ratios for malate and succinate in carcass fatty acids (Figure 2) are about the same as for labeled lactate and glycerol and 6-labeled glucose, but definitely lower than for 1-labeled glucose (Shreeve *et al.*, 1967). There are no significant differences between lean and obese mice in the ^{3}H:^{14}C ratios for carcass fatty acids of mice given labeled malate or succinate.

Because of the relatively high amount of tritium transfer from DL-isocitrate-2-^{3}H and the relatively low amount of carbon-14 transfer from DL-isocitrate-5,6-^{14}C, the carcass fatty acids for this pair-labeled compound have a ^{3}H:^{14}C ratio (Figure 2) which is of a different order of magnitude than for malate or succinate or, indeed, any of the previously used carbohydrates. The ^{3}H:^{14}C ratio for carcass fatty acids is of the same order as for liver fatty acids in the case of la-

[5] In an earlier publication (Shreeve, 1965) lower ratios for malate were reported. Subsequent studies with later preparations of DL-malate-2-^{3}H of higher specific activity have shown higher values for ^{3}H incorporation. Preliminary tests have indicated that addition of malate carrier decreases incorporation of DL-malate-2-^{3}H into fatty acids and water. Deterioration of DL-malate-2-^{3}H, which may occur upon long storage in frozen solution, could be another possible reason for the earlier results.

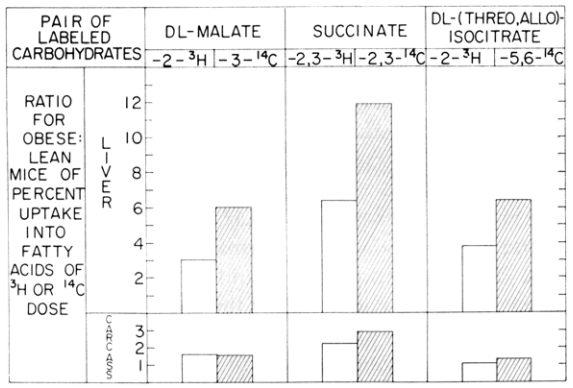

FIGURE 1: Comparisons between lean and obese mice of incorporation of ^{14}C and tritium into fatty acids.

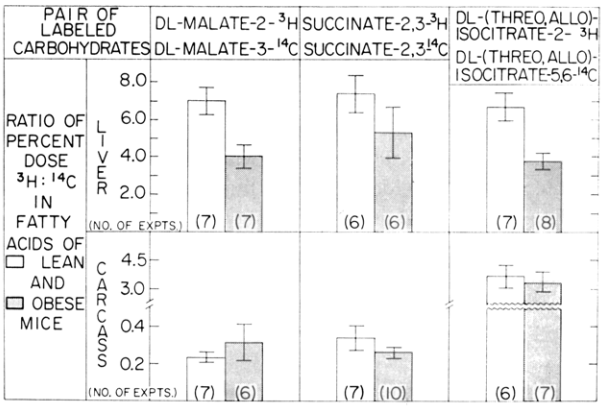

FIGURE 2: Incorporation of tritium relative to ^{14}C from doubly labeled carbohydrates into fatty acids of lean and obese mice.

beled isocitrate. In this respect, also, isocitrate resembles 1-labeled glucose more than it does the other carbohydrates.

Conversion of Tritium-Labeled Carbohydrates into Body Water. Results of measurement of the tritium distilled from blood water *in vacuo* at room temperature have been used together with values for body water space obtained previously (Shreeve *et al.*, 1967) to calculate apparent conversions of tritium-labeled carbohydrates to total body water. Such calculations for DL-malate-2-^3H have provided estimates generally over 100% as indicated in Table II. Extensive search had not disclosed any procedural error as a cause for these unreal high values. Further measurement with injected ^3HOH of body water space in lean and obese mice has not suggested any significant revision of previous estimates. Investigation is in progress to detect the possible occurrence of some metabolic product of DL-malate-2-^3H (perhaps an aldehyde) which is volatile *in vacuo* and which, unlike water, may be confined to the blood volume, *e.g.*, by attachment to plasma proteins. At present, the values in Table II cannot be meaningfully interpreted in regard to water actually formed from DL-malate-2-^3H. A few values over 100% were also encountered with succinate-2,3-^3H so that the mean values for per cent dose of this compound converted into body water (Table II) are also questionable and probably represent at least a slight overestimate.

Much lower values were found for DL-isocitrate-2-^3H, which, of course, would be expected if the *allo* form is inert in the body. As found with some compounds (glucose-1-^3H and DL-lactate-2-^3H) tested earlier, the obese mice seem to show slightly greater conversion of isocitrate to body water than do lean mice. However, the relative amounts of the *threo*-D and -L forms converted are unknown.

Conversion of ^{14}C-Labeled Carbohydrates into Expired Carbon Dioxide. The amount of $^{14}CO_2$, in per cent of ^{14}C dose administered, which was formed and excreted in the breath up to 90 min after intraperitoneal injection, is given in Table II. From DL-malate-2-^{14}C about 50% appears as $^{14}CO_2$ in both lean and obese mice. If D-α-hydroxy acid dehydrogenase (Tubbs, 1965) is operative on the D-malate-3-^{14}C, some of the ^{14}C may derive from that isomer. If the same proportions of the $^{14}CO_2$ derive from the D and L isomers as in the case of DL-lactate-2-^{14}C, then a lesser fraction of natural (L) malic acid is oxidized to $^{14}CO_2$ than of natural (L) lactic acid (Shreeve *et al.*, 1967). Whereas from glucose-6-^{14}C, glycerol-1,3-^{14}C, and (probably) DL-lactate-2-^{14}C less $^{14}CO_2$ was formed in obese than in lean mice (Shreeve *et al.*, 1967), from DL-malate-3-^{14}C and from succinate-2,3-^{14}C (Table II) the $^{14}CO_2$ formed was about the same in obese as in lean mice. This suggests that the general impairment in oxidation of carbohydrates at the level of pyruvate or above in obesity and diabetes (Shreeve *et al.*, 1968) does not extend to the level of the tricarboxylic acid cycle acids. The amount of $^{14}CO_2$ formed from succinate-2,3-^{14}C was greater than from DL-malate-3-^{14}C and about the same as was formed from DL-lactate-2-^{14}C.

With DL-isocitrate-5,6-^{14}C the lesser amount of about 25% of dose converted into $^{14}CO_2$ again probably reflects the inertness of one-half of the administered mixture of isomers. As with the other DL isomers (malate and lactate) the proportions of $^{14}CO_2$ deriving from D-*threo*- and L-*threo*-isocitrate are unknown. In any case, no difference is seen between lean and obese mice in the amounts converted into $^{14}CO_2$.

Discussion

The present study suggests that, in percentage of total dose incorporated into fatty acids and in relation to ^{14}C-labeled analogs, DL-malate-2-^3H and succinate-2,3-^3H are in the same general category as DL-lactate-2-^3H and glycerol-2-^3H. Like the latter two compounds, malate and succinate show as much or more tritium transferred to fatty acids of liver than of carcass, whereas by contrast glucose-1-^3H shows far greater total transfer in extrahepatic tissues than in liver (Shreeve *et al.*, 1967). In terms of tritium theoretically available for transfer to pyridine nucleotides (*cf.* Results) malate and succinate generally exceed lactate in percentage of available dose incorporated.

The latter expression cannot readily be related in the *in vivo* situation to molar quantities of labeled substrate transferred to fatty acids. Since concentrations of lactate in tissues and plasma are 10–100 times those of malate and succinate (Frohman *et al.*, 1951), a similar extent of labeling would seem to indicate a far greater molar contribution from lactate. However, turnover rates for the pools of these compounds at the synthetic site are not known, and differences in these rates could offset differences in specific activity of sub-

strates. There could be sharper pulse labeling for compounds (such as dicarboxylic acids) with higher turnover rates.

No definite conclusion can be made, therefore, as to whether the reducing protons (tritium) of lactate, or of succinate or glycerol, are in each case transferred to fatty acids *via* malate through coupling of various NAD^+-linked reactions to L-malate:$NADP^+$ oxidoreductase (malic enzyme) (Lowenstein, 1961a; Pande *et al.*, 1964; Young *et al.*, 1964; Ball, 1966; Shreeve *et al.*, 1967). However, the present findings do allow for this possibility, since minor differences in labeling by various substrates could be explained, *e.g.*, by differing efficiencies of uptake by the liver, turnover rates, or isotope discrimination.

If tritium is transferred to fatty acids from succinate-2,3-^{3}H only *via* formation of L-malate-2,3-^{3}H it is somewhat curious that the percentage dose of ^{3}H incorporated from succinate is as high as (lean mice) or greater than (obese mice) from DL-malate-2^{3}H and that the ^{3}H:^{14}C ratio in liver fatty acids is as high for succinate as for malate. These two dicarboxylic acids would be expected to have similar fates except that succinate is initially metabolized intramitochondrially, whereas dehydrogenases for malate occur in both mitochondria and cytosol (Shrago and Lardy, 1966). L-Malate-2,3-^{3}H formed from tritiated succinate, therefore, should be more available for disposition through mitochondrial oxidation and less available for transfer *via* cytoplasmic enzymes to fatty acids. The relatively high labeling from succinate-2,3-^{3}H (in the liver fatty acids of obese mice almost 7% of that tritium theoretically available for transfer to pyridine nucleotides (*cf.* Results)) indicates a high rate of transfer of intramitochondrial malate to the cytoplasm, as has been suggested to occur as an important mechanism for transfer of reducing equivalents between the two cellular compartments (Shrago and Lardy, 1966). A recent study with ^{14}C-labeled succinate and isolated rat heart mitochondria (McElroy *et al.*, 1968) supports this concept. If the studies with rat liver slices (Foster and Bloom, 1963) correctly indicate a major exchange of the protons of water with the reducing proton of NADPH during fatty acid synthesis, then the efficiency of transfer of the tritium of labeled malate from mitochondrion to cytoplasm to NADPH would be exceedingly high. Differences between liver slices and liver *in situ* may be suspected, as has been discussed for the case of ^{3}H:^{14}C ratios in fatty acids formed from glucose-6-^{3}H-6-^{14}C (Shreeve *et al.*, 1967).

In another instance, *i.e.*, the extent of labeling of glucose in the intact rat from injected DL-malate-2-^{3}H and succinate-2,-3-^{3}H (Oji and Shreeve, 1967), there is also higher (by two- to threefold) incorporation of tritium from succinate than from malate, with a distinctly higher ^{3}H:^{14}C ratio for succinate. Differences between malate and succinate in response of glucose labeling to glucocorticoid treatment (Oji and Shreeve, 1967) would further suggest a compartmental separation of exogenous malate from that malate which originates from succinate within the mitochondrion.

Not completely ruled out is the possibility that the tritium which is removed from succinate-2,3-^{3}H in the initial dehydrogenation is transferred to an intramitochondrial pool of NADH *via* $FADH_2$ (Ernster and Lee, 1964; Chance *et al.*, 1965). However, as described in the Results section, characteristics of this system indicate that little, if any, tritium would accompany the reducing equivalents in transfer to fatty acids. Intramitochondrial fatty acids synthesized by the "chain elongation pathway" would presumably be the recipients of this tritium, if at all (Whereat, 1965). Preliminary evidence that tritium from succinate-2,3-^{3}H is distributed into C-16 fatty acids of the liver almost as much as into C-18 (Lamdin *et al.*, 1965) suggests that the *de novo* synthetic system of the cytoplasm is the major pathway for transfer of tritium from succinate to fatty acids.

The carbon-14 of malate and succinate could presumably be converted into fatty acids by formation of oxaloacetate, successive decarboxylation (in the mitochondrion) of oxaloacetate to acetyl-CoA, conversion of the latter into citrate, migration of citrate from mitochondrion to cytoplasm, cleavage of citrate to oxaloacetate and acetyl-CoA, and incorporation of acetyl-CoA into fatty acids. Thereby, after the initial oxidations, there is a common (mitochondrial) pathway. Generally similar patterns and extent of ^{14}C incorporation into liver and carcass fatty acids for DL-malate-3-^{14}C and succinate-2,3-^{14}C suggest the occurrence of such a common pathway.[6]

Earlier studies (Shreeve *et al.*, 1967) did not show any significant differences among ^{3}H- or ^{14}C-labeled glucose, lactate, or glycerol in the O:L ratio of incorporation into liver or carcass fatty acids. The present results with malate, succinate, and isocitrate do seem to suggest some variability among the carbohydrates and the type of isotopic label within a given carbohydrate. In particular, with malate-2-^{3}H and isocitrate-2-^{3}H in liver fatty acids, there are relatively low O:L ratios and correspondingly low ^{3}H:^{14}C ratios for these labeled carbohydrates in obese compared with lean mice. On the other hand, the O:L ratio for succinate-2,3-^{14}C in liver fatty acids of obese mice is uniquely high. Possibly such findings could indicate special characteristics of obese mice for metabolism of these carbohydrates, but further evidence is needed to confirm the validity of these interesting differences.

Transfer of ^{14}C from DL-isocitrate-5,6-^{14}C to fatty acids can occur only *via* conversion into citrate (by aconitase) and cleavage by citrate lyase to form acetyl-CoA-1-^{14}C (Spencer and Lowenstein, 1962). Since the O:L ratio is no greater than for other compounds, an increase in citrate lyase (Kornacker and Lowenstein, 1965) does not seem to be a dominant factor in explanation of the hyperlipogenic phenomenon revealed generally in the obese mice by all of the labeled compounds. However, when the values for per cent of total dose incorporated into fatty acids from DL-isocitrate-5,6-^{14}C (*threo* plus *allo* isomers) are corrected by factors relating to the active isomer involved, and the one out of two labeled carbons incorporated, then the per cent of "active" dose converted to fatty acids is relatively high in the liver, higher perhaps than for malate or succinate, though not higher than for DL-lactate-2-^{14}C (Shreeve *et al.*, 1967). In contrast to the liver, incorporation into carcass fatty acids from DL-isocitrate-5,6-^{14}C is very much lower than from ^{14}C-labeled malate or succinate or any of the previously studied compounds. Rather than indicating any low activity of citrate lyase (which is very high in adipose tissue of rats (Ball, 1966)), the results may signify some other characteristic of nonhepatic tissues, such as relatively low aconitase activity.

[6] Spencer and Lowenstein (1962) raised the possibility of a different pathway by noting that labeled carbons 5 and 6 of citrate (carboxyl carbons of oxaloacetate formed by citrate lyase) were incorporated into fatty acids by mammary gland homogenates to an extent equal to that for labeled carbons 3 and 4 of citrate (carbons 2 and 3 of derived oxaloacetate).

Adequate delivery of isocitrate to metabolic sites in nonhepatic tissues is indicated by a relatively high labeling of carcass fatty acids by DL-isocitrate-2-^{3}H. Again, considering "active" doses involved, the per cent of ^{3}H transferred to carcass fatty acids from this compound well exceeds that of any other ^{3}H-labeled carbohydrates so far investigated in these studies. Some time ago, an important role was envisioned for isocitrate dehydrogenase in the biosynthesis of fatty acids (Lowenstein, 1961b). As an $NADP^+$-linked cytoplasmic enzyme present in high activity, it appeared to have characteristics strategically suitable as a source of reducing hydrogen for fatty acid synthesis. Yet, unlike glucose 6-phosphate dehydrogenase and L-malate:$NADP^+$ oxidoreductase, this enzyme does not increase under conditions favoring lipogenesis (Pande *et al.*, 1964; Leveille and Hanson, 1966). The present data suggest a substantial contribution of reducing hydrogen from isocitrate so far as extrahepatic tissues are concerned. For liver, on the other hand, isocitrate seems to be a relatively minor source of reducing hydrogen. The present findings with ^{3}H-labeled malate and succinate, together with the earlier data with ^{3}H-labeled lactate and glycerol, on the whole suggest that reducing hydrogen for fatty acid biosynthesis in the liver derives predominantly from a variety of NAD^+-linked substrates, presumably in conjunction with a transhydrogenation mechanism for generating NADPH. Our data are consistent with earlier suggestions (Pande *et al.*, 1964; Young *et al.*, 1964) that L-malate:$NADP^+$ oxidoreductase ("malic enzyme") is a key enzyme in the channel for delivery of hydrogen from various sources to fatty acids of the liver.

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