Metabolism of [1,3-^{13}C]Glycerol-1,2,3-Tris(methylsuccinate) and Glycerol-1,2,3-Tris(methyl[2,3-^{13}C]succinate) in Rat Hepatocytes

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Hepatocytes prepared from overnight-fasted rats were incubated for 120 minutes in the presence of 2.5 mmol/L [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate) or glycerol-1,2,3-tris(methyl[2,3-^{13}C]succinate). The identification and quantification of ^{13}C-enriched metabolites by a recently developed method for the deconvolution of nuclear magnetic resonance (NMR) spectra with multiplet structures and constraints documented a virtually complete recovery of [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate) in ^{13}C-labeled glycerol, lactic acid, and glucose. In hepatocytes exposed to [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate), glucose was symmetrically labeled, with the vast majority of hexose molecules being enriched with ^{13}C on both C_1 and C_3 and/or C_6 and C_4. The respective abundance of glucose isotopomers labeled either on both C_3 and C_4 or on only 1 of these 2 C atoms indicated that the triose phosphates generated from [1,3-^{13}C]glycerol represented 44% ± 1% of the total amount of triose phosphates incorporated into the hexose. In hepatocytes exposed to glycerol-1,2,3-tris(methyl[2,3-^{13}C]succinate), the recovery of [2,3-^{13}C]succinate, [2,3-^{13}C]fumarate, and either double- or single-labeled malate, lactate, alanine, and glucose accounted for about half the initial ^{13}C content of the ester. The majority of the glucose molecules were now labeled in both C_1 and C_2 or C_6 and C_5, with a preferential labeling of C_6-C_5 relative to C_1-C_2, the paired C_6/C_1 and C_5/C_2 ratios averaging 1.33 ± 0.04. These findings show that glycerol-1,2,3-tris(methylsuccinate) is efficiently and extensively metabolized in hepatocytes. They reinforce the concept that the asymmetry of glucose ^{13}C-labeling by triose phosphates generated from Krebs cycle intermediates is modulated by the availability of glycerol-derived triose phosphates. Lastly, the present study indicates that the latter triose esters, under the present experimental conditions which do not aim at duplicating the physiological in vivo situation, are largely directly channelled in the gluconeogenic pathway, with only a limited intrahepatic contribution of the "indirect" pathway involving their back-and-forth interconversion to and from pyruvate.

SELECTED ESTERS of succinic acid are currently under investigation as tools to bypass site-specific defects in the metabolism of circulating nutrients, with the eventual aim of using these esters either as insulinotropic agents in non–insulin-dependent diabetes mellitus or as alternative nutrients in starvation, endotoxemia, or organ transplantation.[1-4] In this perspective, novel esters of succinic acid with increased nutritional value were recently synthesized.[5-7] The major aim of the present study was to explore, by ^{13}C nuclear magnetic resonance (NMR), the metabolic fate of one of these new esters, glycerol-1,2,3-tris(methylsuccinate), in rat hepatocytes.

MATERIALS AND METHODS

The method used for the synthesis of ^{13}C-labeled glycerol-1,2,3-tris(methylsuccinate) was adapted from the method used for the synthesis of ^{14}C-labeled ester.[8] The labeled products were identified by ^{1}H and ^{13}C NMR spectroscopy on a Bruker AM300 spectrometer (Bruker Analytic GmbH, Rheinstelten, Germany). Spectra were recorded in $CDCl_3$, and chemical shifts are reported in ppm downfield from tetramethylsilane (s, d, and m for singlet, doublet, and multiplet, respectively). The following 4 compounds were prepared.

[1,3-^{13}C]Glycerol-1,2,3-Tris(methylsuccinate)

N-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (489 mg, 2.55 mmol) was added at 0°C to a mixture of [1,3-^{13}C]glycerol (26 mg, 0.277 mmol, 99 atom % ^{13}C; Isotec, Miamisburg, OH), dry dichloromethane (11 mL), monomethylsuccinate (334 mg, 2.53 mmol), and 4-(dimethylamino)-pyridine (34 mg, 0.278 mmol), and the mixture was stirred at 0°C for 1 hour and at room temperature for 80 minutes. 1,2-Diaminoethane (167 μL, 2.52 mmol) was added and stirring continued for 1 hour. Ethyl acetate (33 mL) was added and the mixture was washed with 0.1 mol/L hydrochloric acid (2 × 10 mL), saturated aqueous sodium hydrogen carbonate (2 × 10 mL), and brine (10 mL), dried over magnesium sulfate, and concentrated in a vacuum. The residue was chromatographed on silica gel (2 g, 63 to 200 μm) with ether to yield [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate) (113 mg, 94%). Proton (^{1}H) NMR data: δ 5.29 (m, 1H), 4.34 (m, 2H), 4.22 (m, 2H), 3.70 (s, 9H), and 2.64 (m, 12H). ^{13}C NMR δ 62.3 (~90× above natural abundance).

[2,3-^{13}C]Succinic Anhydride

[2,3-^{13}C]succinic acid (99 mg, 0.825 mmol, 99 atom % ^{13}C; Cambridge Isotope Laboratories, Woburn, MA) was refluxed with 0.5 mL acetyl chloride (bath temperature 79°C) for 3 hours. The precipitate obtained upon cooling in an ice bath was washed with ice-cold ether (2 × 1 mL) and dried to yield [2,3-^{13}C]succinic anhydride (68 mg, 81%; melting point, 118°C).

Monomethyl-[2,3-^{13}C]Succinate

[2,3-^{13}C]succinic anhydride (68 mg, 0.667 mmol) was dissolved in methanol (20 mL) and kept at 65°C in a stoppered flask for 170 minutes. The solvent was removed under vacuum to yield monomethyl-[2,3-^{13}C]succinate (82 mg, 92%).

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Glycerol-1,2,3-Tris(methyl-[2,3-^{13}C]succinate)

N-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (175 mg, 0.913 mmol) was added at 0°C to a mixture of anhydrous glycerol (15.8 mg, 0.172 mmol), dry dichloromethane (6.7 mL), monomethyl-[2,3-^{13}C]succinate (82 mg, 0.612 mmol), and 4-(dimethylamino)-pyridine (21 mg, 0.172 mmol), and the mixture was stirred at 0°C for 1 hour and at room temperature for 80 minutes. 1,2-Diaminoethane (100 µL, 1.5 mmol) was added and stirring continued for 1 hour. Ethyl acetate (20 mL) was added and the mixture was washed with 0.1 mol/L hydrochloric acid (6 mL and 12 mL), saturated aqueous sodium hydrogen carbonate (2 × 6 mL), and brine (6 mL), dried over magnesium sulfate, and concentrated in a vacuum. The residue was chromatographed on silica gel (1.2 g, 40 to 63 µm) with ether to yield glycerol-1,2,3-tris(methyl-[2,3^{13}C]succinate) (68 mg, 90%). ^{1}H NMR δ 5.28 (m, 1H), 4.33 (dd, 4.3 Hz, 11.9 Hz, 2H), 4.20 (dd, 5.9 Hz, 11.9 Hz, 2H), 3.70 (s, 9H), and 2.65 (m, 12H). ^{13}C NMR δ 29.6 to 28.3 m (~90× above natural abundance).

Two groups of 2 × 10^8 hepatocytes prepared by the collagenase perfusion method[9] from each of 3 overnight-fasted female Wistar rats (175 ± 3 g; Proefdierencentrum, Heverlee, Belgium) were incubated for 120 minutes at 37°C in a HEPES and bicarbonate–buffered medium[10] containing bovine serum albumin (1.0 mg/mL) and either [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate) or glycerol-1,2,3-tris-(methyl[2,3-^{13}C]succinate) (2.5 mmol/L) and equilibrated against a mixture of O_2 and CO_2 (19/1 vol/vol). The volume of incubation medium and hepatocytes was 10 mL.

After incubation and centrifugation, the supernatant medium was stored at −20°C overnight and lyophilized. The cell pellet was extracted with 3 mL of a solution of perchloric acid (5% vol/vol). This extract was neutralized by addition of 1.5 to 2.0 mL KOH (2 mol/L), mixed with 50 mL ethanol for glycogen precipitation, and centrifuged. The extract was then lyophilized.

The procedures used for ^{13}C NMR data acquisition and analysis are identical to methods described elsewhere.[11]

The recovery of HEPES in the incubation medium and cell pellet, as judged by its C_1 peak area, was 63.6 ± 8.5 and 4.1 ± 1.5 µmol (n = 6), with comparable results being obtained for the C_2 to C_6 resonances of the molecule.

All results are presented as the mean ± SEM, together with the number of individual observations (n) when different from 3. All ratios mentioned in the text were calculated from paired measurements in each individual experiment. The statistical significance of differences between mean values was assessed by Student's *t* test.

The symbols α-G1 and β-G1 refer to the α- and β-anomer of [1-^{13}C]glucose. Comparable symbols were used for the other isotopomers of the hexose. Symbols such as α,β-G4 or α-G2,5 refer to pairs of anomers or isotopomers with overlapping ^{13}C resonance that prevents their individual quantification. The letters s and d refer to single- and double-labeled metabolites, respectively.

RESULTS

Fate of [1,3-^{13}C]Glycerol-1,2,3-Tris(methylsuccinate)

For experiments in the presence of [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate), about half of the initial amount of the ester (25 µmol) was recovered as nonesterified [1,3-^{13}C]-glycerol (Table 1). The paired ratio between the cell/medium content of [1,3-^{13}C]glycerol was 8.3% ± 1.7% (n = 3). No [2-^{13}C]glycerol signal was observed.

The 2 sole metabolites of [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate) that were found were lactic acid and glucose.

Except for 1 of 6 measurements, no [2-^{13}C]lactate was observed. Virtually identical readings were recorded for the $^{13}C_1$ and $^{13}C_3$ signals of lactate in the incubation medium or cell extract. They yielded mean values for [1,3-^{13}C]lactate of 562 ± 117 and 36 ± 23 nmol (n = 6 in both cases) in the incubation medium and cell extract, respectively.

Table 1. Nonglucidic Metabolites (µmol) Generated by Rat Hepatocytes Exposed to Glycerol-1,2,3-Tris(methylsuccinate) 2.5 mmol/L

Substrate Material	[1,3-^{13}C]Glycerol-1,2,3-Tris(methylsuccinate)		Glycerol-1,2,3-Tris(methyl[2,3-^{13}C]succinate)	
	Incubation Medium	Cell Extract	Incubation Medium	Cell Extract
[1,3-^{13}C]glycerol	12.13 ± 3.30	0.99 ± 0.31		
[2,3-^{13}C]succinate			7.12 ± 1.20	0.78 ± 0.12
[2,3-^{13}C]fumarate			3.72 ± 0.29	0.88 ± 0.10
[2-^{13}C]malate				
(s)			0.08 ± 0.02	0.01 ± 0.01
(d)			16.92 ± 1.51	0.58 ± 0.18
[3-^{13}C]malate				
(s)			0.85 ± 0.09	Nil
(d)			14.54 ± 1.25	0.51 ± 0.22
[1-^{13}C]lactate				
(s)	0.54 ± 0.11	0.03 ± 0.03	Nil	Nil
(d)	Nil	Nil	Nil	Nil
[2-^{13}C]lactate				
(s)	Nil	0.01 ± 0.01	0.25 ± 0.17	0.04 ± 0.02
(d)	Nil	Nil	2.93 ± 0.26	0.39 ± 0.05
[3-^{13}C]lactate				
(s)	0.58 ± 0.23	0.04 ± 0.04	0.27 ± 0.10	0.06 ± 0.03
(d)	Nil	Nil	2.54 ± 0.19	0.38 ± 0.02
[2-^{13}C]alanine				
(s)			0.02 ± 0.02	Nil
(d)			0.64 ± 0.18	Nil
[3-^{13}C]alanine				
(s)			0.04 ± 0.04	Nil
(d)			0.57 ± 0.09	Nil

The total amount of ^{13}C-labeled D-glucose recovered in both the incubation medium and cell extract, expressed as the [1,3-^{13}C]glycerol equivalent, was 7.60 ± 0.68 μmol.

The measurement of [1,3-^{13}C]glycerol, [1,3-^{13}C]lactic acid, and ^{13}C-labeled D-glucose yielded a total production of 21.3 ± 3.4 μmol [1,3-^{13}C]glycerol equivalent, a value not significantly different ($P > .35$) from the nominal initial content in the incubation medium of [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate) (25.0 μmol). Such measurements do not include the amount of ^{13}C-labeled D-glucose possibly incorporated into glycogen.

For the most abundant single-labeled (s) species, the output of ^{13}C-labeled glucose was 91.3% ± 1.0% (n = 21) of the total production of each isotopomer. This percentage does not cover the data for α-G2,5 and β-G2, in which cases the total production was too low to allow a sizable identification of the isotopomer in the cell extract (Table 2).

Except as otherwise mentioned, all further data are restricted to measurements made in the incubation medium.

The mean paired α-G1/β-G1 ratio (53.3% ± 2.5%; n = 3) and α-G6/β-G6 ratio (55.6% ± 1.7%, n = 3) for the (s) species of ^{13}C-labeled glucose were virtually identical to one another, with an overall mean value of 54.5% ± 1.4% (n = 6) and hence a relative abundance of about 35.3% and 64.7% for α- and β-D-glucose, respectively.

The resonance area of the $^{13}C_4$ signal assigned to double-labeled α,β-G4 was not significantly different from the sum of the areas attributed to double-labeled α-G3 and β-G3,5 as judged by group ($P > .6$) or paired ($P > .3$) comparison. These measurements yielded a mean value for glucose molecules labeled on both C_3 and C_4 of 1.52 ± 0.22 μmol (n = 6).

In the case of the (s) species of glucose, there was no significant difference between the amount of α-G1 and α-G6 (paired α-G6/α-G1 ratio, 1.05 ± 0.09), β-G1 and β-G6 (paired β-G6/β-G1 ratio, 0.95 ± 0.09), and α-G3 + β-G3 and α,β-G4 (paired α,β-G4/α-G3 + β-G3 ratio, 0.91 ± 0.07), yielding an overall mean value for the labeling of the C_4-C_5-C_6/C_3-C_2-C_1 moieties of the hexose of 0.97 ± 0.05 (n = 9). This again indicates a lack of asymmetry in such a labeling process. In these calculations, the amount of the (s) species of β-G3 was taken as the mean of the following 2 independent estimations: β-G3 = α-G3 × β/α anomeric ratio (ie, 1.84) and β-G3 = β-G3,5 − the estimated amount of β-G5 (calculated from half of the α-G2,5 values and the β/α anomeric ratio). Incidentally, there was no significant difference between these 2 estimations as computed from measurements made in the incubation medium or cell extract, with a mean paired ratio between the first and second estimation of 1.07 ± 0.10 (n = 6).

There was also no significant difference between the amount of the (s) species of α-G1 (or α-G6) and the sum of the (d) and (s) species of α-G3 (or α-G4), with a mean paired ratio of 1.04 ± 0.07 (n = 6) between these 2 entities. Likewise, the (s) species of β-G1 (or β-G6) was comparable to the sum of the (d) species of β-G3,5 and (s) species of β-3 (or sum of the (d) and (s) species of β-G4), with a mean paired ratio of 1.01 ± 0.05 (n = 6). This indicates that virtually all of the molecules of

Table 2. Glucose Isotopomers (nmol) Generated by Hepatocytes Exposed to Glycerol-1,2,3-Tris(methylsuccinate) 2.5 mmol/L

Substrate Material	[1,3-^{13}C]Glycerol-1,2,3-Tris(methylsuccinate)		Glycerol-1,2,3-Tris(methyl-[2,3-^{13}C]succinate)	
	Incubation Medium	Cell Extract	Incubation Medium	Cell Extract
α-G1				
(s)	1,227 ± 310	133 ± 36	160 ± 46	Nil
(d)	Nil	Nil	640 ± 147	26 ± 26
α-G2,5				
(s)	103 ± 40	Nil	Nil	Nil
(d)	Nil	Nil	1,492 ± 298	182 ± 91
α-G3				
(s)	679 ± 114	90 ± 55	Nil	Nil
(d)	592 ± 186	Nil	Nil	Nil
α-G6				
(s)	1,243 ± 209	116 ± 22	52 ± 25	5 ± 5
(d)	Nil	Nil	827 ± 153	78 ± 39
β-G1				
(s)	2,334 ± 647	276 ± 34	277 ± 56	29 ± 29
(d)	Nil	Nil	1,180 ± 231	100 ± 100
β-G2				
(s)	132 ± 26	Nil	82 ± 29	Nil
(d)	Nil	Nil	1,112 ± 243	169 ± 91
β-G3,5				
(s)	1,517 ± 379	118 ± 74	160 ± 80	48 ± 25
(d)	789 ± 213	Nil	1,412 ± 292	246 ± 123
β-G6				
(s)	2,139 ± 476	213 ± 31	328 ± 29	5 ± 5
(d)	Nil	Nil	1,604 ± 233	321 ± 66
α,β-G4				
(s)	1,851 ± 407	123 ± 78	NM	NM
(d)	1,663 ± 257	Nil	NM	NM

Abbreviation: NM, not measurable.

newly formed D-glucose were labeled on both C_1 and C_3 and/or both C_6 and C_4.

In fair agreement with such a proposal, no glucose labeled with ^{13}C on C_2 or C_5 was found in the cell extract. In the incubation medium, the total amount of such isotopomers, all of the (s) species, did not exceed 0.33 ± 0.07 μmol. This corresponds to the sum of α-G2,5 (0.10 ± 0.04 μmol), β-G2 (0.13 ± 0.03 μmol), and β-G5 (0.10 ± 0.04 μmol), the latter amount being estimated from the values for α-G2,5 and the anomeric ratio (n = 3 in all cases).

For the purpose of comparison, the total amount of the (s) species of α-G3 (0.68 ± 0.11 μmol), β-G3 (1.33 ± 0.28 μmol), and α,β-G4 (1.85 ± 0.41 μmol) recovered in the incubation medium was 3.86 ± 0.51 μmol, ie, more than 10 times the value found for the (s) species labeled with ^{13}C on C_2 and/or C_5.

The mean value for the generation of glucose labeled with ^{13}C on both C_3 and C_4, ie, the (d) species of the hexose, when divided by the paired mean value for the generation of glucose labeled solely on C_3 or C_4, ie, the (s) species of the sugar, was 78.7% ± 1.0% (n = 3). This percentage corresponds to the $m^2/[m(1 - m)]$ ratio, in which m and (1 − m) represent the relative abundance of ^{13}C-labeled and unlabeled triose phosphates, respectively. Thus, m was 44.0% ± 0.3% (n = 3), indicating that in the hepatocytes exposed to [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate), the triose phosphates generated from the glycerol moiety of the ester were less abundant than the unlabeled triose phosphates originating from other precursors.

Fate of Glycerol-1,2,3-Tris(methyl[2,3-^{13}C]succinate)

The amount of double-labeled [2,3-^{13}C]succinate and [2,3-^{13}C]fumarate residues recovered in the incubation medium was 7.12 ± 1.20 and 3.72 ± 0.29 μmol, respectively (Table 1).

Doublet signals corresponding to double-labeled [1,2-^{13}C]malate and [3,4-^{13}C]malate were also identified. However, their precise quantification was not judged to be reliable because of the low signal to noise ratio. Such was also the case for single-labeled [1-^{13}C]malate and [4-^{13}C]malate.

In the case of the single-labeled (s) species of ^{13}C-labeled malate, the net generation of [3-^{13}C]malate (0.86 ± 0.09 μmol) largely exceeded ($P < .005$) that of [2-^{13}C]malate (0.08 ± 0.02 μmol). However, such was not the case for the much more abundant double-labeled (d) species. Thus, as expected, the integrated areas of the $^{13}C_2$ and $^{13}C_3$ signals assigned to [2,3-^{13}C]malate were not significantly different from one another, with an overall mean value of 15.73 ± 1.02 μmol (n = 6).

The production of double-labeled [2,3-^{13}C]lactate also largely exceeded that of single-labeled lactate isotopomers. Again, the integrated areas of the $^{13}C_2$ and $^{13}C_3$ signals ascribed to [2,3-^{13}C]lactate were not significantly different from one another in the incubation medium or cell extract. Likewise, the production of [2,3-^{13}C]alanine, which yielded comparable $^{13}C_2$ and $^{13}C_3$ signals, was about 1 order of magnitude higher than that of the (s) species of the amino acid. Except in 1 record, the amount of single-labeled [2-^{13}C]lactate or [2-^{13}C]alanine was lower than that of the corresponding [3-^{13}C]metabolites, with a mean paired [2-^{13}C]/[3-^{13}C] ratio of 27.3% ± 10.6% ($P < .025$ *v* unity).

The data presented thus far all refer to measurements made in the incubation medium. The content of the cell extract, when expressed relative to the total production of each metabolite, was 10.4% ± 2.4% in the case of [2,3-^{13}C]succinate, 19.3% ± 2.5% for [2,3-^{13}C]fumarate, 3.6% ± 1.0% for [2,3-^{13}C]malate, and 12.5% ± 1.1% for [2,3-^{13}C]lactate.

The output and cell content of ^{13}C-labeled glucose averaged, respectively, 1,058 ± 130 and 86 ± 39 nmol for the (s) species of the hexose and 4,135 ± 501 and 561 ± 91 nmol for the (d) species (Table 2). When expressed as [2,3-^{13}C]succinate equivalent, the total production of ^{13}C-labeled D-glucose was 5,260 ± 514 nmol. This represented only 7.0% ± 0.7% of the succinate residues (75 μmol) present in the initial amount of extracellular glycerol-1,2,3-tris(methyl[2,3-^{13}C]succinate).

The data presented thus far indicate that the integrated amount of ^{13}C-labeled succinate, fumarate, malate, lactate, alanine, and glucose recovered in both the incubation medium and cell extract was 38.56 ± 1.71 μmol [2,3-^{13}C]succinate equivalent, representing 51.4% ± 2.3% of the initial ester content in succinate residues. However, these data do not cover all ^{13}C-labeled metabolites. For instance, the production of $^{13}CO_2$ and possible generation of ^{13}C-labeled glycogen were not included in our measurements.

In the case of the more abundant (d) species, the output of ^{13}C-labeled D-glucose was 90.9% ± 1.7% (n = 21) of the paired total amount of the same isotopomer recorded in both the incubation medium and cell extract. Such a percentage is virtually identical ($P > .8$) to that found for the (s) species of ^{13}C-labeled glucose in experiments in the presence of [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate).

When expressed as a triose phosphate equivalent, containing 1 or 2 ^{13}C, the total output of ^{13}C-labeled glucose from hepatocytes exposed to glycerol-1,2,3-tris(methyl[2,3-^{13}C]succinate) was 5.19 ± 0.52 μmol. Compared with the total output of ^{13}C-labeled glucose generated from [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate), ie, 7.07 ± 0.67 μmol [1,3-^{13}C]glycerol equivalent, the former value was lower ($P < .05$) than expected (9.00 ± 0.86 μmol) from the m value previously mentioned. Such a difference is likely attributable to the production of unlabeled triose phosphates generated both from endogenous nutrients and via metabolism of the succinate moiety of glycerol-1,2,3-tris(methyl[2,3-^{13}C]succinate) and to the limited generation, from the same ester, of some ^{13}C-labeled glucose isotopomers that could not be quantified in a reliable manner because of overlapping resonances (eg, between α,β-G4 and [2-^{13}C]malate).

In hepatocytes exposed to glycerol-1,2,3-tris(methyl[2,3-^{13}C]succinate), the mean paired α-G1/β-G1 ratio (53.4% ± 3.6%; n = 3) and α-G6/β-G6 ratio (51.6% ± 2.2%; n = 3) for the most abundant (d) species were not significantly different ($P > .3$) from the mean values found in cells exposed to [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate).

In the (d) series, all of the mean paired ratios for the ^{13}C resonance areas of α-G1 + α-G6/α-G2,5, β-G1/β-G2, or β-G6/β-G3,5 were not significantly different from unity, with an overall mean value of 1.07 ± 0.04 (n = 9). This indicates that the (d) species of G1 and G2, as well as those of G6 and G5, were actually the same glucose molecules labeled in both C_1 and C_2 and/or C_6 and C_5. This is consistent with the absence of double-labeled G3 isotopomers.

In the cell extract, the total amount of the (d) species of α-G1, β-G1, α-G6, and β-G6 (0.53 ± 0.13 μmol) was also close to the total amount of the (d) species of α-G2,5, β-G2, and β-G3,5 (0.59 ± 0.15 μmol).

However, in the (s) series, the β-G1/β-G2 and β-G6/β-G3,5 paired ratios largely exceeded unity. Thus, even after the exclusion of 1 extremely high value, the ratio was 2.98 ± 0.63 ($n = 5$, $P < .04$). Likewise, the amount of (s) species of α-G1 + α-G6 exceeded the negligible value for the (s) species of α-G2,5. This indicates a more efficient labeling of C_1 (or C_6) than C_2 (or C_5) when only 1 carbon in each pair of atoms (C_1-C_2 and C_6-C_5) is labeled with ^{13}C.

In the case of the (d) species, all individual α-G6/α-G1, β-G6/β-G1, and β-G3,5/β-G2 paired ratios exceeded unity, with an overall mean value of 1.33 ± 0.04 ($n = 9$, $P < .001$). A comparable situation prevailed in the cell extracts, in which the amount of double-labeled α-G1, β-G1, and β-G2 was below the level of detection in 5 of 9 cases. In the other 4 cases, the just-mentioned paired ratios were 1.44 ± 0.10 ($P < .025$ *v* unity). In the case of the much less abundant (s) species, the trend was also for a more pronounced ^{13}C-labeling of C_6-C_5-C_4 than C_1-C_2-C_3 in both the incubation medium and cell extract, but this difference failed to achieve statistical significance.

Figure 1 shows that the values for G1,2 and G6,5 (4.13 ± 0.50 μmol), G1 and G6 (0.82 ± 0.08 μmol), G2 and G5 (0.24 ± 0.10 μmol), and G3 and G4 (nil) were grossly proportional to those found in a prior study[11] under comparable experimental conditions in the presence of the dimethyl ester of [2,3-^{13}C]succinic acid (10.0 mmol/L). Thus, for the 3 former pairs of isotopomers, the mean results obtained in the present study averaged 74.7% ± 8.2% of those recorded in the prior investigation. The ^{13}C-labeling of glucose thus appeared proportional to the initial concentration of extracellular esterified succinate residues, ie, 7.5 mmol/L in the present study and 10.0 mmol/L in the former one.

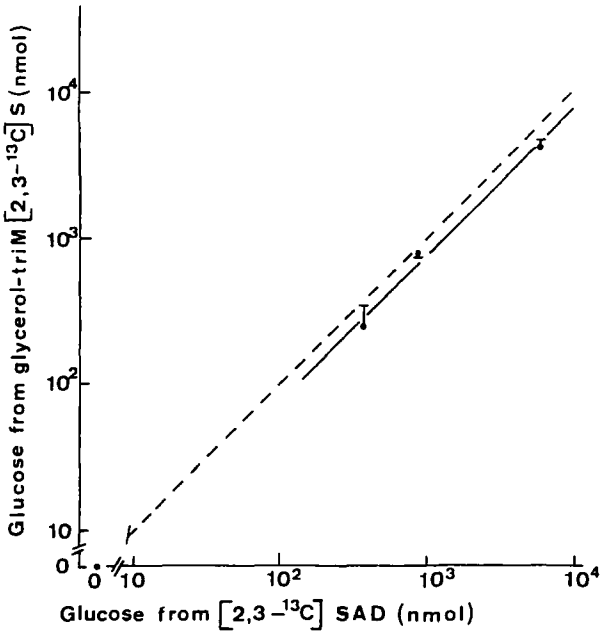

Fig 1. Mean values (mean ± SEM) for the production of ^{13}C-labeled glucose by hepatocytes exposed to glycerol-1,2,3-tris-(methyl[2,3-^{13}C]succinate) (2.5 mmol/L, glycerol-triM[2,3-^{13}C]S) are compared with the mean production of the ^{13}C-labeled hexose by hepatocytes incubated in the presence of the dimethyl ester of [2,3-^{13}C]succinic acid (10.0 mmol/L, [2,3-^{13}C]SAD). Both series of results are expressed as nmol and ranged on logarithmic scales. The 4 points in this figure, from left to right, refer to the generation of G3 and G4, G2 and G5, G1 and G6, and G1,2 and G6,5. The broken oblique line corresponds to a theoretical identity between the 2 series of measurements; the solid oblique line indicates that the production of the different isotopomers of glucose from glycerol-1,2,3-tris-(methyl[2,3-^{13}C]succinate) only averaged 74.7% ± 8.2% of the corresponding values found with [2,3-^{13}C]succinate dimethyl ester.

DISCUSSION

The present findings document that glycerol-1,2,3-tris(methylsuccinate) is efficiently metabolized in rat hepatocytes. In hepatocytes exposed to glycerol-1,2,3-tris(methyl[2,3-^{13}C]succinate), about half of the initial amount of the ester was recovered in ^{13}C-labeled succinate, fumarate, malate, lactate, alanine, and glucose, a situation comparable to that previously found under comparable conditions in hepatocytes incubated in the presence of the dimethyl ester of [2,3-^{13}C]succinic acid.[11] In the present study, the amount of [2,3-^{13}C]succinate recovered in the cell extract and incubation medium averaged 7.90 ± 1.22 μmol, ie, no more than about 10% of the succinate residues initially present in the ester (75 μmol). However, the amount of [1,3-^{13}C]glycerol recovered in the incubation medium and cell extract in experiments in the presence of [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate) averaged 13.12 ± 3.51 μmol, ie, about half of the initial glycerol content of the ester.

Compared with the results of a prior study[12] in the presence of [U-^{14}C]glycerol-1,2,3-tris(methylsuccinate) or glycerol-1,2,3-tris(methyl[2,3-^{14}C]succinate), the production of ^{13}C-labeled acidic metabolites, amino acids, and extracellular D-glucose, if expressed per cell, followed a comparable hierarchy, averaging, for instance, 20.5% ± 4.9% and 26.6% ± 2.6% of prior values in the case of the ^{13}C-labeled acidic metabolites and extracellular D-glucose formed from [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate), and 28.0% ± 1.6% and 23.2% ± 2.5% in the case of the ^{13}C-labeled acidic metabolites and extracellular D-glucose formed from glycerol-1,2,3-tris(methyl[2,3-^{13}C]succinate). The lower absolute values for the production of metabolites in the present versus the former study is probably related to the fact that the number of cells relative to the volume of incubation medium was more than 10 times higher in the present experiments versus those performed with the ^{14}C-labeled ester.

As already mentioned, the present results for the production of ^{13}C-labeled D-glucose isotopomers from glycerol-1,2,3-tris(methyl[2,3-^{13}C]succinate) are also in fair agreement with those obtained in hepatocytes exposed to the dimethyl ester of [2,3-^{13}C]succinic acid,[11] provided that allowance is made for the difference in the initial concentration of extracellular esterified succinate residues in the 2 series of experiments. Likewise, the present study confirms that in hepatocytes exposed to an ester of [2,3-^{13}C]succinic acid, the generation of the (d) species of malate, lactate, and alanine largely exceeds that of the corresponding (s) species, and in the latter case, the production of [3-^{13}C]isotopomers exceeds that of [2-^{13}C]isotopomers.[11] As discussed elsewhere,[11] these findings can be accounted for by the metabolic fate of [2,3-^{13}C]succinate during successive turns in the Krebs cycle.

The present results are relevant to 2 current issues concerning hepatic gluconeogenesis and glycogen synthesis. The first of these issues relates to the mechanism responsible for the asymmetrical labeling of D-glucose generated in hepatocytes from ^{13}C-labeled precursors such as pyruvate.[13-15] There is no doubt that such an asymmetry is linked to the isotopic dilution of ^{13}C-labeled dihydroxyacetone phosphate by unlabeled molecules generated from endogenous nutrients such as triglyceride-derived glycerol. However, it apparently remains a matter of debate as to whether the lack of isotopic equilibration between the pools of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate implies a process of enzyme-to-enzyme channeling.

When ^{13}C-labeled D-glucose was generated from [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate), the 2 moieties of the hexose (C_1-C_2-C_3 and C_4-C_5-C_6) were equally enriched with ^{13}C atoms. This finding indicates that the free pools of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate displayed a comparable relative enrichment in ^{13}C-labeled molecules, and hence argues against the view that the intrinsic catalytic properties of triose phosphate isomerase are inadequate to ensure such an isotopic equilibrium.

However, when ^{13}C-labeled D-glucose was generated from glycerol-1,2,3-tris(methyl[2,3-^{13}C]succinate), the C_4-C_5-C_6 moiety of the hexose was more efficiently labeled than its C_1-C_2-C_3 moiety, with a paired ratio of 1.33 ± 0.04 for the glucose molecules released in the incubation medium. To our knowledge, this is the most marked asymmetry thus far observed for the ^{13}C-labeling of D-glucose generated by hepatocytes in fasted rats or humans,[14-17] as expected from the simultaneous generation of large amounts of triose phosphates from both the succinate and glycerol moieties of glycerol-1,2,3-tris(methylsuccinate).

Although the time course for ^{13}C-labeling of D-glucose does not need to be identical in hepatocytes exposed to [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate) and glycerol-1,2,3-tris(methyl[2,3-^{13}C]succinate), the comparison of the 2 series of results strongly suggests that ^{13}C-labeled D-glyceraldehyde-3-phosphate generated from the succinate moiety of the ester undergoes enzyme-to-enzyme channeling between glyceraldehyde-3-phosphate dehydrogenase and phosphofructoaldolase.[14,15]

Figure 2 indeed documents that the experimental values both for the molar amount of ^{13}C-labeled D-glucose formed from either the glycerol moiety of [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate) or the succinate moiety of glycerol-1,2,3-tris(methyl[2,3-^{13}C]succinate) and for the ratio in ^{13}C-labeling of the C_4-C_5-C_6/C_1-C_2-C_3 moieties of D-glucose could be reproduced in a model including the postulated process of enzyme-to-enzyme channeling. In this model, allowance is made for the lower labeling of the C_1-C_2-C_3 moiety of D-glucose 6-phosphate resulting from its circulation in the pentose phosphate pathway. The model also takes into account the fact that the enzyme-to-enzyme channeling of D-glyceraldehyde-3-phosphate between glyceraldehyde-3-phosphate dehydrogenase and phosphofructoaldolase implies a lesser incorporation of free (as distinct from enzyme-bound) D-glyceraldehyde-3-phosphate than free dihydroxyacetone phosphate into D-glucose in molecules generated from [2,3-^{13}C]succinate or [1,3-^{13}C]glycerol.

In hepatocytes from fasted rats incubated for 45 minutes at fixed concentrations of lactate (1.0 mmol/L) and pyruvate (0.1 to 0.2 mmol/L) and increasing concentrations of glycerol

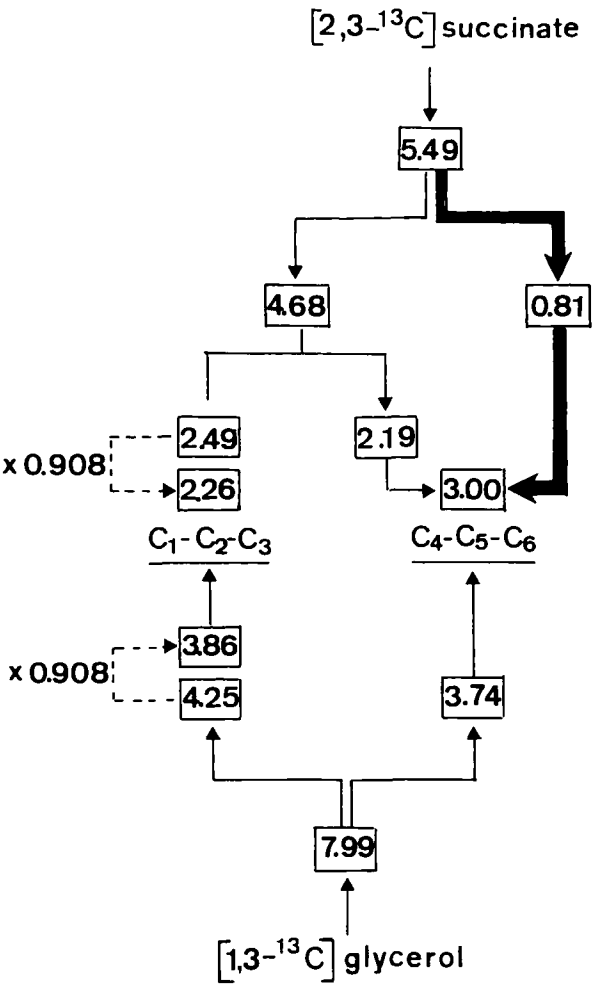

Fig 2. Schematic view for the ^{13}C-labeling of the 2 moieties of D-glucose generated by hepatocytes exposed to [1,3-^{13}C]glycerol-1,2,3-tris(methyl[2,3-^{13}C]succinate). The enzyme-to-enzyme channeling of D-glyceraldehyde-3-phosphate between glyceraldehyde-3-phosphate dehydrogenase and phosphofructoaldolase is assumed to be 0.81 μmol (heavy arrow). It leads to a 12% fractional decrease in the incorporation of free D-glyceraldehyde-3-phosphate into de novo formed D-glucose relative to the incorporation of free dihydroxyacetone phosphate, ie, from 4.25 to 3.74 μmol for triose phosphates formed from [1,3-^{13}C]glycerol and from 2.49 to 2.19 μmol for triose esters produced from [2,3-^{13}C]succinate. Hence, the total fluxes from D-glyceraldehyde-3-phosphate (3.00 + 3.74 = 6.74 μmol) and from dihydroxyacetone phosphate (2.49 + 4.25 = 6.74 μmol) into D-glucose are identical. The circulation in the pentose phosphate pathway results in a decrease of the ^{13}C content of the C_1-C_2-C_3 moiety of D-glucose to 90.8% of the value that would prevail in the absence of such circulation, ie, from 4.25 to 3.86 μmol for hexose molecules formed from [1,3-^{13}C]glycerol and from 2.49 to 2.26 μmol for those generated from [2,3-^{13}C]succinate (broken arrows). In this scheme, the total amount of ^{13}C-labeled D-glucose is 7.60 μmol (3.86 + 3.74) for hexose molecules formed from 1,3-^{13}C]glycerol and 5.26 μmol (2.26 + 3.00) for hexose molecules formed from [2,3-^{13}C]succinate, values similar to the experimental results. Likewise, in the present scheme, the C_4-C_5-C_6/C_1-C_2-C_3 ratio for ^{13}C-labeled D-glucose is equal to 0.97 (ie, 3.74/3.86) for hexose molecules formed from [1,3-^{13}C]glycerol and 1.33 (ie, 3.00/2.26) for glucidic molecules formed from [2,3-^{13}C]succinate, ratios that are again identical to the mean experimental values.

(0.025, 0.04, and 0.3 mmol/L), Previs et al[18] observed that the triose phosphate equilibrium in cells exposed to [2-^{13}C]glycerol, ie, the ratio of ^{13}C enrichment in the C_4-C_6/C_1-C_3 moieties of D-glucose, progressively increases from 89% to 95%. However, in these experiments, the triose phosphate equilibrium in cells exposed to [3-^{13}C]lactate and [3-^{13}C]pyruvate, ie, the ratio of ^{13}C enrichment in the C_1-C_3/C_4-C_6 moieties of D-glucose, progressively decreased from 89% to 77%. These results were considered in the context of the zonation of selected enzyme activities. In essence, the present findings confirm these observations, since the asymmetry of D-glucose labeling was obvious in cells exposed to glycerol-1,2,3-tris(methyl[2,3-^{13}C]succinate) and not detectable in cells exposed to [1,3-^{13}C]glycerol-tris(methylsuccinate). Thus, both the study by Previs et al[18] and the present study clearly document that in the same cell preparation, the isotopic equilibrium of triose phosphates is vastly different for molecules generated from either glycerol or precursors entering the gluconeogenic pathway via phosphoenol pyruvate. Both series of observations are indeed compatible with the process of enzyme-to-enzyme channeling depicted in Fig 2.

The second point of discussion concerns the possible back-and-forth intrahepatic interconversion of triose phosphates and pyruvate.[19] In this respect, it should first be duly underlined that the present experiments did not aim at duplicating the physiological situation normally prevailing in vivo. Hence, the information obtained in this study should in no way be taken as indicative of the modalities of gluconeogenesis in vivo. With these remarks in mind, the present data indicate that the labeling of both C_1 and C_3 (and/or C_4 and C_6) of D-glucose formed by hepatocytes exposed to [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate) does not rule out the view that the ^{13}C-labeled triose phosphates generated from this precursor are first converted to pyruvic acid, the latter 2-ketoacid then serving as a gluconeogenic precursor. In such a case, the de novo generation of isotopomers of D-glucose could be calculated from the metabolic scheme illustrated in Fig 3 in a manner comparable to that proposed in a prior study in hepatocytes exposed to [2,3-^{13}C]succinic acid dimethyl ester.[11] Hence, it could be calculated that the generation of G2,5 by hepatocytes exposed to [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate) would represent about 36% of the total amount of glucose molecules labeled on C_1 and/or C_6. Actually, our results indicate that the production of G2,5 represented only about 5% of the amount of C_1- and/or C_6-labeled glucose generated by hepatocytes exposed to [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate). The "indirect" pathway for gluconeogenesis, namely that involving back-and-forth interconversion between triose phosphates and pyruvate, thus appears as a limited, albeit not entirely negligible, modality for the de novo synthesis of glucose from [1,3-^{13}C]glycerol-1,2,3-tris(methylsuccinate) under the present experimental conditions.

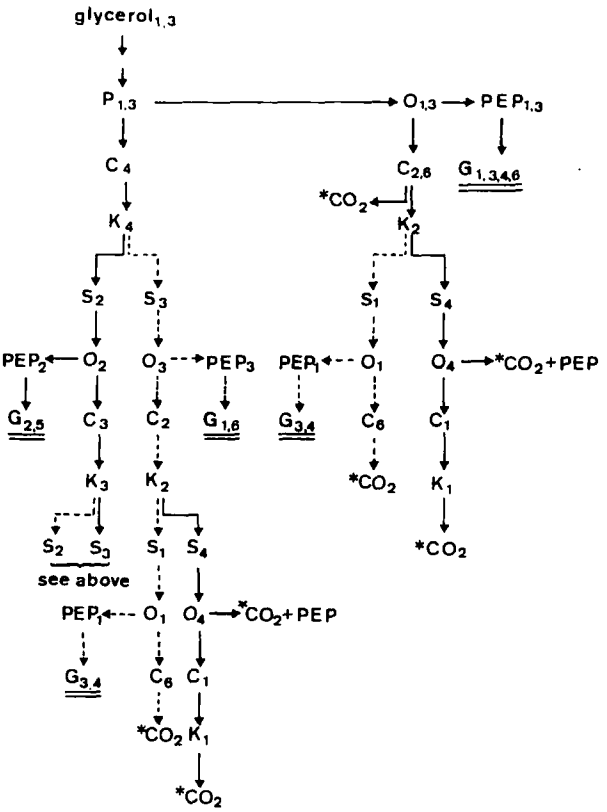

Fig 3. Schematic view for the conversion of [1,3-^{13}C]pyruvate ($P_{1,3}$), generated from [1,3-^{13}C]glycerol (glycerol$_{1,3}$), to either citrate (C) via the pyruvate dehydrogenase and citrate synthase reactions or oxaloacetate (O) via the pyruvate carboxyloase reaction, followed by the generation of 2-ketoglutarate (K), succinate (S), phosphoenol pyruvate (PEP), and glucose (G). The site of ^{13}C-labeling is indicated as a subscript, with the numbering of the carbon atoms being identical in S and O. Labeled CO_2 is indicated by an asterisk. Broken arrows refer to reactions that would not occur if the enzyme-to-enzyme tunneling of succinyl CoA to malate were fully efficient.

In conclusion, while documenting that glycerol-1,2,3-tris(methylsuccinate) represents a most efficient gluconeogenic precursor in rat hepatocytes, the present study also provides evidence in support of the enzyme-to-enzyme channeling of D-glyceraldehyde-3-phosphate between glyceraldehyde-3-phosphate dehydrogenase and phosphofructoaldolase, and against an extensive back-and-forth intrahepatic interconversion of triose phosphates and pyruvate.

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