Effects of Starvation and Diabetes on the Metabolism of [2,3-^{13}C]Succinic Acid Dimethyl Ester in Rat Hepatocytes

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The metabolism of [2,3-^{13}C]succinic acid dimethyl ester ([2,3-^{13}C]-SAD) 10 mmol/L was examined in hepatocytes from overnight-fasted normal rats, 3-day starved rats, and overnight-fasted hereditarily diabetic Goto-Kakizaki (GK) rats. The amount of ^{13}C-labeled succinate, fumarate, malate, lactate, alanine, and aspartate released by the hepatocytes was much higher in fasted normal rats than in starved or diabetic animals. Although the integrated areas of the $^{13}C_2$ and $^{13}C_3$ signals assigned to double-labeled malate, lactate, or alanine were not significantly different, the amount of single-labeled malate, lactate, alanine, and aspartate was higher in C_3- versus C_2-labeled isotopomers. The release of ^{13}C-labeled glucose by the hepatocytes was lower in fasted versus starved or diabetic rats. Virtually all hexose molecules double-labeled in the C_1-C_2-C_3 and/or C_6-C_5-C_4 moieties corresponded to the [1,2-^{13}C] and/or [5,6-^{13}C] isotopomers. However, in the case of the single-labeled species, ^{13}C-labeling of C_1 (or C_6) exceeded that of C_2 (or C_5). Both the single- and double-labeled molecules enriched with ^{13}C in the C_1-C_2-C_3 moiety were less abundant than those labeled in the C_6-C_5-C_4 moiety, with such asymmetry being most marked in overnight-fasted normal rats, less pronounced in diabetic animals, and virtually absent in starved rats. These findings document that SAD is efficiently metabolized in hepatocytes, with its use as a gluconeogenic precursor being influenced by the nutritional and hormonal status of the animals. The present experiments also reinforce the view that asymmetrical labeling of glucose by ^{13}C-labeled precursors is modulated by the relative contribution of exogenous and endogenous nutrients to the production of triose phosphates incorporated into the hexose.

THE DIMETHYL ESTER of succinic acid (SAD) is currently under investigation as a potential tool to prevent adenosine triphosphate depletion in cells endangered by an imbalance between the generation and utilization of this nucleotide. This nutrient is indeed efficiently metabolized in several cell types, including pancreatic islet cells,[1] hepatocytes,[2] myocytes,[3] neural cells,[4] colonocytes,[5] and adipocytes.[6] It was shown to prevent or minimize the metabolic consequences of starvation[7,8] and experimental endotoxemia[9] and to display insulinotropic action in both normal and diseased pancreatic islet cells.[10-12] It offers the considerable advantage over unesterified succinic acid to efficiently cross the plasma membrane. It then undergoes intracellular hydrolysis, so that succinic acid eventually becomes readily available for mitochondrial metabolism. Thus, SAD is oxidized at a much higher rate than succinic acid tested at an equimolar concentration.[1] SAD may also bypass site-specific defects in the metabolism of circulating nutrients, eg, at the level of D-glucose transport or phosphorylation.[1]

In the present study, a novel computational strategy for the deconvolution of nuclear magnetic resonance (NMR) spectra with multiplet structures and constraints[13] was used to compare the metabolism of [2,3-^{13}C]SAD in hepatocytes from overnight-fasted normal rats, 3-day starved animals, and overnight-fasted Goto-Kakizaki (GK) rats, with the latter animals considered a model of hereditary non–insulin-dependent diabetes mellitus.[14]

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MATERIALS AND METHODS

[2,3-^{13}C]succinic acid was purchased from ARC Laboratories (Amsterdam, The Netherlands). Its dimethyl ester ([2,3-^{13}C]SAD) was synthesized by a procedure similar to that previously designed for the synthesis of ^{14}C-labeled SAD.[1]

Groups of hepatocytes (2×10^8 each) were prepared by the collagenase perfusion method[15] from either overnight-fasted female Wistar rats (Proefdierencentrum, Heverlee, Belgium), 3-day starved animals of the same sex and source, and overnight-fasted female GK rats from our local colony. The body weight of overnight-fasted normal and GK rats was comparable, with an overall mean weight of 243 ± 17 g versus 186 ± 3 g ($P < .05$) for the 3-day starved animals. The hepatocytes were incubated for 120 minutes at 37°C in a HEPES- and bicarbonate-buffered medium[16] containing bovine serum albumin (1.0 mg/mL) and [2,3-^{13}C]SAD (10 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 10.0 mL. After incubation and centrifugation, the supernatant was stored at −20°C overnight and then lyophilized.

The procedures for ^{13}C-NMR data acquisition and analysis are described elsewhere.[13]

All results are presented as the mean ± SEM. Unless stated otherwise, all data refer to four individual observations. 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. (s) and (d) refer to single- and double-labeled metabolites, respectively.

RESULTS

The amount of HEPES recovered in the incubation medium was not significantly different in experiments with hepatocytes from overnight-fasted normal rats (fasted), 3-day starved normal rats (starved), and overnight-fasted GK rats (GK), with an overall mean value from the C_1 signal of 61.96 ± 4.31 μmol (n = 12).

Table 1. Output of ^{13}C-Labeled Nonglucidic Metabolites (μmol) From Rat Hepatocytes Incubated for 120 Minutes in the Presence of [2,3-^{13}C]SAD (10 mmol/L)

Metabolite	Fasted	Starved	GK
[2,3-^{13}C]succinate	3.76 ± 1.50	0.08 ± 0.08	0.15 ± 0.05
[2,3-^{13}C]fumarate	6.86 ± 0.59	4.48 ± 0.70	3.83 ± 0.29
[2-^{13}C]malate			
(s)	0.11 ± 0.07	0.40 ± 0.31	0.31 ± 0.18
(d)	33.97 ± 4.05	21.17 ± 2.96	17.89 ± 0.85
[3-^{13}C]malate			
(s)	3.23 ± 1.14	1.06 ± 0.25	0.86 ± 0.20
(d)	29.76 ± 3.50	17.06 ± 2.78	15.39 ± 1.02
[2-^{13}C]lactate			
(s)	0.14 ± 0.12	Nil	Nil
(d)	2.86 ± 0.20	1.00 ± 0.53	1.58 ± 0.23
[3-^{13}C]lactate			
(s)	0.18 ± 0.05	0.06 ± 0.05	0.14 ± 0.02
(d)	2.83 ± 0.29	0.99 ± 0.65	1.37 ± 0.14
[2-^{13}C]alanine			
(s)	0.19 ± 0.06	Nil	Nil
(d)	0.92 ± 0.24	0.54 ± 0.22	1.52 ± 0.32
[3-^{13}C]alanine			
(s)	0.02 ± 0.01	Nil	0.13 ± 0.08
(d)	0.80 ± 0.14	Nil	1.14 ± 0.12

The amount of [2,3-^{13}C]succinate (d species) present in the incubation medium was higher ($P < .005$) in fasted versus starved and GK rats (Table 1). Such was also the case for [2,3-^{13}C]fumarate ($P < .005$).

The integrated areas of the $^{13}C_2$ and $^{13}C_3$ signals assigned to [2,3-^{13}C]malate were not significantly different in any of the three groups of rats. For the output of [2,3-^{13}C]malate, the mean values were 31.86 ± 2.58, 19.12 ± 2.03, and 16.64 ± 0.78 μmol in fasted, starved, and GK rats, respectively (n = 8 in all cases). Once again, the production of this metabolite was thus higher ($P < .005$) in fasted versus starved or GK rats.

In the case of the single-labeled (s) species of ^{13}C-labeled malate, the net generation of [3-^{13}C]malate was greater ($P < .001$) than that of [2-^{13}C]malate. Thus, relative to the total amount of these two single-labeled ^{13}C metabolites, the amount of [3-^{13}C]labeled dicarboxylic acid was 83.8% ± 6.3% (n = 12). The mean total amount of [2-^{13}C]malate and [3-^{13}C]malate (s species) released in the incubation medium was higher ($P < .06$) in fasted versus starved and GK rats.

The pattern for the generation of ^{13}C-labeled isotopomers of lactate was comparable to that just described for ^{13}C-labeled malate. First, in the case of the double-labeled (d) species of [2,3-^{13}C]lactate, the integrated areas of the $^{13}C_2$ and $^{13}C_3$ signals were not significantly different. Second, when enough of the (s) species of lactate was produced to allow reliable quantification, the amount of [3-^{13}C]lactate was 89.2% ± 7.5% (n = 8) of the total amount of both [2-^{13}C]lactate and [3-^{13}C]lactate. Third, the mean production of both the (d) and (s) species of ^{13}C-labeled lactate was higher in fasted versus starved or GK rats, with the difference achieving statistical significance ($P < .001$ in both cases) only in the case of the more abundant (d) species.

Likewise, the much smaller amounts of double-labeled [2,3-^{13}C]alanine were not significantly different when judged by the integrated areas of the $^{13}C_2$ and $^{13}C_3$ signals. As a rule, an insufficient amount of the (s) species of [2-^{13}C]alanine and [3-^{13}C]alanine was produced to allow reliable comparisons between these isotopomers in the three groups of rats.

In fasted but not in starved or GK rats, minor amounts of double-labeled [2,3-^{13}C]aspartate (0.23 ± 0.10 μmol, n = 8) and single-labeled [3-^{13}C]aspartate (0.05 ± 0.03 μmol, n = 4) but no [2-^{13}C]aspartate were also found in the incubation medium.

The total amount of nonglucidic ^{13}C-labeled metabolites released by the hepatocytes in the incubation medium, expressed as [2,3-^{13}C]SAD equivalents, was 48.28 ± 3.43 μmol in fasted rats, compared with only 26.22 ± 3.72 and 24.37 ± 0.85 μmol in starved and GK rats, respectively ($P < .005$).

Generation of D-Glucose

The α/β anomeric ratio, as judged from the readings for the (d) species of α-G1 and β-G1 and/or α-G6 and β-G6, was 52.0% ± 0.8%, 52.2% ± 3.2%, and 49.3% ± 3.8% in fasted, starved, and GK rats, respectively.

In fasted rats, the total output of ^{13}C-labeled D-glucose expressed as [2,3-^{13}C]SAD equivalents was 6.80 ± 0.74 μmol (n = 4), as compared with 19.36 ± 2.45 and 10.61 ± 0.42 μmol (n = 4 in both cases) in starved and GK rats, respectively ($P < .005$). Relative to the total generation of ^{13}C-labeled metabolites, generation of D-glucose represented 12.6% ± 1.8% in starved rats versus 43.3% ± 7.1% and 30.4% ± 1.1% in starved and GK rats, respectively (Table 2).

In the case of (d) species of D-glucose generated by hepato-

Table 2. Output of ^{13}C-Labeled Glucose Isotopomers (nmol) From Rat Hepatocytes Incubated for 120 Minutes in the Presence of [2,3-^{13}C]SAD (10 mmol/L)

Isotopomer	Fasted	Starved	GK
α-G1			
(s)	95 ± 17	393 ± 79	268 ± 55
(d)	936 ± 164	3,372 ± 484	1,801 ± 57
α-G2,5			
(s)	102 ± 38	Nil	Nil
(d)	2,610 ± 184	5,554 ± 816	3,360 ± 347
α-G3			
(s)	Nil	Nil	Nil
(d)	138 ± 62	Nil	Nil
α,β-G4			
(s)	Nil	Nil	Nil
(d)	Nil	Nil	Nil
α-G6			
(s)	242 ± 135	198 ± 52	71 ± 33
(d)	1,457 ± 229	2,988 ± 281	1,561 ± 185
β-G1			
(s)	227 ± 72	764 ± 159	490 ± 54
(d)	1,803 ± 322	5,805 ± 619	3,147 ± 109
β-G2			
(s)	113 ± 61	401 ± 99	26 ± 18
(d)	1,612 ± 282	5,005 ± 595	2,641 ± 187
β-G3,5			
(s)	154 ± 12	931 ± 289	529 ± 66
(d)	1,967 ± 351	5,920 ± 641	3,087 ± 114
β-G6			
(s)	333 ± 103	869 ± 178	465 ± 105
(d)	1,950 ± 300	6,521 ± 847	3,774 ± 89

cytes from fasted rats, the paired (α-G1 + α-G6)/ α-G2,5 ratio (92.4% ± 7.3%), β-G1/β-G2 ratio (111.8% ± 3.4%), and β-G6/ β-G3,5 ratio (100.7% ± 6.8%), as a rule, were not significantly different from unity, with an overall mean value of 101.6% ± 4.0% (n = 12). Likewise, in starved and GK rats, the same paired ratios, as a rule, were not significantly different from unity; however, the overall mean values (113.8% ± 2.6% in starved rats and 114.0% ± 4.8% in GK rats, n = 12) were slightly higher ($P < .02$ or less) than 100%. These data indicate that the vast majority of the (d) species of D-glucose were actually labeled with ^{13}C on both C_1 and C_2 and/or C_6 and C_5. This interpretation is supported by the low values for α-G3 and α,β-G4 isotopomers.

However, in the case of the (s) species, ^{13}C-labeling of C_1 (or C_6) exceeded that of C_2 (or C_5). For instance, in fasted rats, the paired (α-G1 + α-G6)/(α-G1 + α-G6 + α-G2,5) ratio (75.3% ± 13.6%), β-G1/(β-G1 + β-G2) ratio (75.8% ± 9.9%), and β-G6/(β-G6 + β-G3,5) ratio (60.0% ± 13.9%) had an overall mean value of 70.3% ± 6.9% (n = 12), significantly larger than ($P < .02$) a theoretical value of 50.0%. Comparable results were recorded in starved and GK rats, in which the overall mean values for the three paired ratios were 73.7% ± 6.0% and 80.6% ± 7.6% (n = 12, $P < .005$ *v* 50.0% in both cases), respectively. Taken as a whole, these results indicate a more efficient labeling of C_1 (or C_6) versus C_2 (or C_5) when only one of the two carbons of C_1-C_2 (or C_6-C_5) is labeled with ^{13}C.

The latter situation was confirmed by the finding that the mean value of the paired (s)/(d) ratios for α-G1, β-G1, α-G6, and β-G6 isotopomers was greater than for the α-G2, β-G2, α-G5, and β-G3,5 isotopomers. In fasted rats, the ratios were 13.8% ± 3.2% (n = 16) in the former case versus 5.8% ± 1.1% (n = 16) in the latter case, ($P < .03$). Likewise, in starved and GK rats, the former ratios were 11.0% ± 0.9% and 12.1% ± 1.6% versus 5.7% ± 1.8% and 4.5% ± 1.9% (n = 16 in all cases) for the latter ratios ($P < .02$ or less).

In fasted rats, the paired α-G1/(α-G1 + α-G6), β-G1/(β-G1 + β-G6), β-G2/(β-G2 + β-G3,5), and α-G2/(α-G2 + α-G5) ratios for the (d) species were 39.5% ± 6.8%, 47.7% ± 1.2%, 45.0% ± 1.5%, and 32.0% ± 3.7%, respectively, for an overall mean value of 41.0% ± 2.4% (n = 16), significantly lower ($P < .005$) than 50.0%. Incidentally, α-G2 was estimated from the β-G2 reading and the mean anomeric α/β ratio, and α-G5 was taken as the difference between the α-G2,5 reading and the calculated value of α-G2. It thus appears that the C_1-C_2-C_3 moiety of D-glucose is less efficiently labeled with ^{13}C than the C_6-C_5-C_4 moiety. Even in the case of the much less abundant (s) species of D-glucose, the mean values for the same four paired ratios were always lower than 50%, but this difference failed to achieve statistical significance. When all available data were pooled together (both d and s species), the mean value (42.0% ± 4.0%, n = 32) for the paired ratios yielded a probability just less than .055.

In starved rats, the four ratios for the (d) species remained lower than 50.0% in 11 of 16 individual cases, but with an overall mean value of 48.2% ± 1.1%, not significantly different from 50.0%. The latter mean value was indeed significantly higher ($P < .01$) than the value recorded in fasted rats (41.0% ± 2.4%). In the case of the (s) species of ^{13}C-labeled D-glucose produced by hepatocytes from starved rats, the paired α-G1/(α-G1 + α-G6), β-G1/(β-G1 + β-G6), and β-G2/(β-G2 + β-G3,5) ratios were 45.4% ± 4.8%, not significantly less than 50.0%. Starvation thus virtually abolished the preferential labeling of the C_6-C_5-C_4 moiety of D-glucose relative to the C_1-C_2-C_3 moiety. Indeed, even when pooling the results obtained in the (s) and (d) series, the overall mean value for the paired ratios (47.1% ± 2.4%, n = 28) remained nonsignificantly lower ($P > .2$) than 50.0%.

Lastly, in GK rats, the α-G1/(α-G1 + αG-6), β-G1/(β-G1 + β-G6), β-G2/(β-G2 + β-G3,5), and α-G2/(α-G2 + α-G5) paired ratios for the (d) species yielded a mean value of 46.4% ± 1.7% (n = 16), between the values found in fasted and starved rats and slightly lower than 50.0% ($P < .06$). Once again, the mean value for the α-G1/(α-G1 + α-G6), β-G1/(β-G1 + β-G6), and β-G2/(β-G2 + β-G3,5) paired ratios in the (s) series yielded a mean value lower, albeit not significantly so, than 50.0%. The overall mean value (45.9% ± 4.2%, n = 28) for the (s) and (d) isotopomers also failed to be significantly lower ($P > .3$) than 50% in GK rats. Thus, in overnight-fasted diabetic rats, as in starved nondiabetic animals, the preferential labeling of the C_6-C_5-C_4 moiety of D-glucose was less pronounced than in overnight-fasted control rats.

DISCUSSION

The present study extends to both 3-day starved rats and hereditarily diabetic animals the study of [2,3-^{13}C]SAD metabolism by isolated hepatocytes, with the results compared against findings in prior experiments[13] in hepatocytes from overnight-fasted normal rats. It provides three essential pieces of information.

First, it confirms that SAD is efficiently catabolized in liver cells.[2] The total generation of ^{13}C-labeled metabolites expressed as [2,3-^{13}C]SAD equivalents was 55.08 ± 3.57, 45.58 ± 1.42, and 34.98 ± 1.00 μmol in fasted, starved, and GK rats, respectively, representing about half or less of the initial amount of the ester (100.0 μmol) present in the incubation medium. Since these values do not include the small amounts of ^{13}C-labeled metabolites recovered inside the hepatocytes at the end of incubation[17] and the output of $^{13}CO_2$,[2] they suggest that the oxidative catabolism of the ester was more important ($P < .05$) in starved versus overnight-fasted animals and even higher ($P < .001$) in GK rats.

Second, the present results unambiguously document that de novo production of ^{13}C-labeled D-glucose from [2,3-^{13}C]SAD was also higher in hepatocytes from starved and GK rats versus fasted animals.

Third, the identification of distinct ^{13}C-labeled isotopomers of both acidic metabolites and glucose documents several features of SAD metabolism that were not readily accessible in experiments performed with the ^{14}C-labeled ester.[2]

In the case of the nonglucidic metabolites, emphasis must be placed on the higher amounts of (d) versus (s) species produced by the hepatocytes and, for the latter species, the larger generation of [3-^{13}C] versus [2-^{13}C] isotopomers. These find-

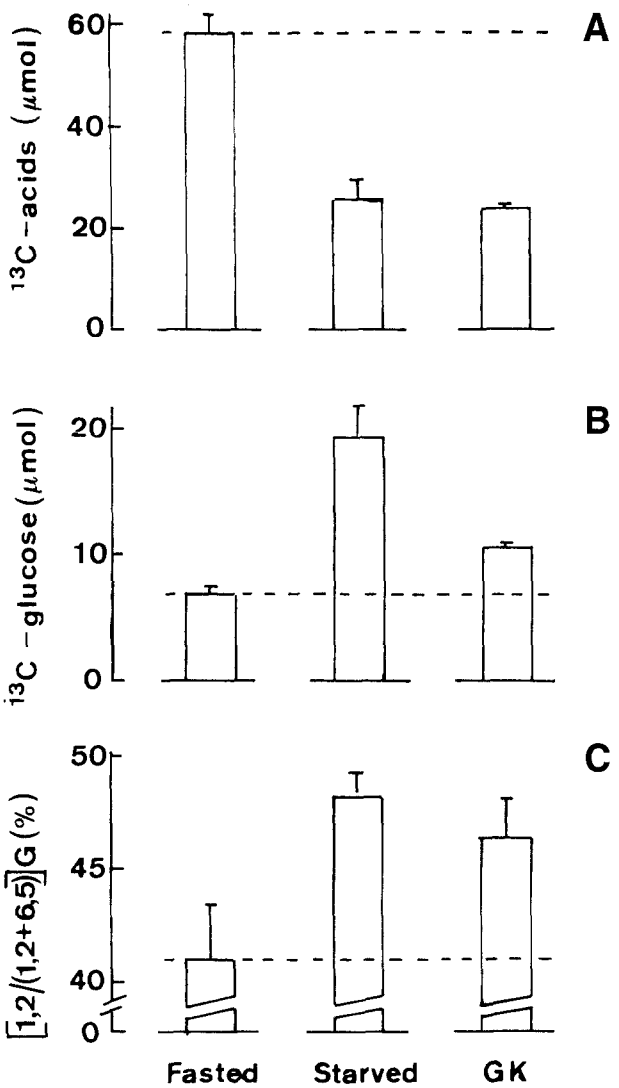

Fig 1. Total generation of ^{13}C-labeled acidic metabolites and amino acids (A) and D-glucose (B) by hepatocytes from fasted, starved, and GK rats exposed to [2,3-^{13}C]SAD. (C) Paired ratio between the (d) species of C_1- and C_2-labeled glucose and the total production of C_1-, C_2-, C_6-, and C_5-labeled hexose. Mean values (±SEM) are derived from 4 (A and B) or 16 (C) individual determinations. (---) Mean values in fasted animals.

ings can be accounted for by the metabolic scheme for SAD metabolism defined in a previous study,[13] especially by the fate of the ester carbon atoms during successive turns of the Krebs cycle.

Likewise, in the case of ^{13}C-labeled glucose, the prevalent production of the (d) species labeled in the C_1-C_2 or C_6-C_5 positions and the more efficient labeling of C_1 (or C_6) versus C_2 (or C_5) of the (s) species can also be accounted for by such a metabolic scheme.

Lastly and most importantly, the finding of an inverse relationship between the rate of gluconeogenesis from [2,3-^{13}C]SAD and the relative extent of asymmetry in glucose ^{13}C-labeling (Fig 1) provides direct support for the view that such asymmetry is dictated by the respective contribution of exogenous and endogenous precursors to the production of triose phosphates eventually incorporated into the hexose.[18] Whether attributable or not to enzyme-to-enzyme channeling between glyceraldehyde-3-phosphate dehydrogenase and phosphofructoaldolase,[19-24] the relative extent of asymmetry would indeed be constant if starvation and diabetes were not associated with both an increase in total gluconeogenesis and a decrease in the availability of endogenous glucogenic precursors.[18]

The present study provides the first information on the metabolic fate of SAD in cells obtained from GK rats, which are currently used as an animal model of non–insulin-dependent diabetes mellitus.[14] It suggests that in vivo administration of SAD could result in an increase of gluconeogenesis in diabetic animals or subjects. This would obviously represent an undesirable situation when considering the possible use of SAD as an insulinotropic tool in the treatment of this disease. However, it should be emphasized that in recent studies a number of novel esters of succinic acid were designed that display a much higher insulinotropic efficiency than SAD.[25] For instance, glycerol-1,2,3-tris(methylsuccinate) administered intravenously to normal rats at a dose as low as 68.8 nmol/g body weight was found to stimulate insulin release and to potentiate the insulinotropic action of the hypoglycemic sulfonylurea gliquidone or the meglitinide analog repaglinide.[26] Such a dose corresponds to no more than 2.8 μg/g body weight or, extrapolated on the theoretical basis of a rule of proportionality, to 1.8 g for a human subject weighing about 65 kg. The latter value is indeed negligible when considering the daily turnover of glucose in the whole body.

However, the ability of SAD to act as a nutrient in hepatocytes may be advantageous in other circumstances. For instance, the viability and metabolic behavior of hepatocytes after liver storage for 20 hours at 4°C was recently found to be improved by incorporation of a succinic acid ester in the Belzer UW-CSS solution (Du Pont Pharma, Wilmington, DE) used for perfusion and storage, suggesting a potential benefit in transplantation procedures.[27]

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