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Preliminary Studies of a Dicarboxylic Acid as an Energy Substrate in Man

G. MINGRONE, R. M. TACCHINO, A. V. GRECO, E. ARCIERI-MASTROMATTEI, F. MARINO, E. FINOTTI, AND E. CASTAGNETO*

From Instituto di Clinica Medica and Instituto di Clinica Chirurgica—Centro C. N.R. per lo studio della Fisiopatologia dello Shock, Università Cattolica S. Cuore, Rome, Italy

ABSTRACT. Azelaic acid (Az), a straight saturated chain nine carbon dicarboxylic acid, was administered in saline form to six healthy male volunteers by iv route. Serum levels of Az and urinary amounts of both azelaic and pimelic (C7) acids were measured by an improved gas liquid chromatographic method. Stoichiometric analysis of Az metabolism was compared with that of glucose and palmitic acid. The respiratory quotient (RQ) as well as the ATP/CO_2 ratio of Az were quite similar to that of palmitic acid. Therefore, Az oxidation is associated with a low cost of ATP synthesis in terms of carbon dioxide production. At the infusion rate used (7.5 g/hr) more than 50% of the administered dose was excreted in the urine. However, the remaining portion was cleared from the plasma in 200 min suggesting an uptake by body tissues which was also confirmed by indirect calorimetric analysis. (*Journal of Parenteral and Enteral Nutrition* **13:**299–305, 1989)

Azelaic acid (Az) is a straight saturated medium-chain dicarboxylic acid with nine carbon atoms, which exhibits a competitive inhibitory action on tyrosinase *in vitro*.[1] For this reason, Az has been effectively used in the treatment of pigmentary disorders of the skin due to hyperfunction of melanocytes, such as melasma, toxic melanoderma,[2] and in the therapy of lentigo maligna.[3] In addition to its therapeutic effect, Az also has a cytotoxic effect on human malignant melanocytes.[4] However, there is experimental evidence that Az does not affect normal human cells[3] and that it has neither a toxic nor a teratogenic effect[5] in laboratory animals.

Recently, it has been shown that dicarboxylic acids (DA) administered to humans or animals can be shortened by units of two carbon atoms during β-oxidation and are only partially excreted in the urine as shorter chain dicarboxylic acids.[6] The β-oxidation of DA can take place in both mitochondria and peroxisomes.[7] The same intermediate metabolites formed during β-oxidation have been found in the two intracellular compartments, although a few enzymes are different.[8–10] Beta-oxidation of DA with an even number of carbon atoms in the peroxisomes ceases at the C6-level and adipic acid (C6) is then liberated into the cytosol where it is absorbed into the mitochondria.[7] The passage of C6 into the mitochondria, where it is oxidized to succinyl-CoA, is independent of carnitine acyl-transferase activity.[11,12] The salts of DA, which are soluble in water, can be directly administered through a peripheral venous route. As in the case of the medium-chain triglycerides (MCT), DA are rapidly oxidized, but they do not require any hydrolysis prior to intracellular utilization. Moreover, as it has been already pointed out, dicarboxylic acids present the advantage of being β-oxidized via a carnitine-independent pathway. This could prove particularly advantageous in certain clinical conditions where a carnitine-deficiency state has been suggested such as in septic processes,[13] liver cirrhosis,[14] premature newborns,[15] and hemodialyzed patients.[16]

Given the present understanding of straight-chain dicarboxylic acid metabolism, the possibility of clinical use of DA in parenteral nutrition (TPN) seems both a feasible and an interesting perspective to explore. In this paper six normal volunteers received an intravenous infusion of 10 g of Az over 80 min. The levels of azelaic and pimelic (C7) acid, the latter being a product of β-oxidation of Az, were measured both in the serum and in the urine of the subjects by an improved gas liquid chromatographic (GLC) method. In addition, Az metabolism was compared to that of glucose and long-chain fatty acids (LCT) by means of indirect calorimetry and stoichiometric analysis to verify if a DA-based parenteral nutrition might have some energy production advantage over glucose or LCT-based parenteral nutrition.

MATERIALS AND METHODS

Azelaic Acid Administration

Six healthy male volunteers, weighing 75 ± 3 Kg, and 40 ± 4 yr old, were given a peripheral iv infusion over 80 min. of 500 ml of double distilled water containing 10 g of Az salified with NaOH, KOH, Mg $(OH)_2$ and Ca $(OH)_2$ in the same proportions at which the Na, K, Mg, and Ca cations are normally present in serum. The infusions were sterilized by a Seitz filter (Staatligh, Berlin, DDR) before administration. The protocol of this study was approved by the Institutional Review Board, Health Science Center of the Catholic University School of Medicine in Rome. Written informed consent was obtained in all cases.

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Reprint requests: Dr. G. Mingrone, Instituto di Clinica Medica, Università Cattolica S. Cuore, Largo A. Gemelli, 8,00168 Roma, Italy.

Serum Collection

Serum samples (5 ml) were collected every 40 min after starting the of infusion, for a period of 200 min. Fifty μg of suberic acid (C8) and sebacic acid (C10) both from Fluka Chemie A.G. (Bughs, Switzerland) were added to 1 ml of each serum sample as internal standards. Sera were acidified to pH 1–2 with 1 N HCl, saturated with NaCl, and extracted three times with eight volumes of ethylacetate. The combined extracts were dried over anhydrous Na_2SO_4 and evaporated under nitrogen stream, and extracted solutes were methylated with diazomethane in diethylether to form dimethyl derivatives.

Urine Samples

The patients voided before starting the Az infusion. Urine samples were collected every 2 hr for the first 10 hr and then in the same container from the 10th to 24th hr. The volume of each sample was measured and 0.5 to 1.0 ml were acidified, extracted, and derivatized as described above.

Gas Chromatographic (GLC) Analysis

Dicarboxylic acid methyl esters were analyzed by gas liquid chromatography (GLC) in a Supelcowax 10 (30 m × 0.32 mm ID, Supelco) silica-fused capillary column fitted to a Hewlett-Packard mod. 5890 A chromatograph. The column temperature was kept at 50°C for 0.5 min, then a gradient from 50°C to 250°C was performed with a rate of 10°C per minute. The injection temperature was 270°C, while that of the detector (FID) was 300°C. The flow rate of high-purity helium, used as the gas carrier, was 2 ml/min. Amounts of 2 μl of the examined samples were injected into the GLC (splitless).

Calibration

One to 100 μg of the standard glutaric (C5), adipic (C6), pimelic (C7), suberic (C8), azelaic (C9), undecandioic (C11), and dodecandioic (C12) acids were esterified with diazomethane; the solvent was evaporated under a nitrogen stream and the residue dissolved in 0.1 ml of hexane. Two μl for each concentration of standard dicarboxylic acid derivatives were injected into the GLC.

Recovery

Ten to 100 μg of C5, C6, C7, C8, C9, C10, C11, and C12 standard DA were added to 1 ml of both the serum and the urine of the untreated subjects. DA were extracted, esterified, and analyzed as described above.

Indirect Calorimetry

Respiratory and metabolic analysis were performed according to a previously developed method,[17–19] which includes: a computerized monitoring system based on a mass spectrometer to rapidly analyze oxygen, carbon dioxide, and nitrogen concentrations; a pneumotachometer to measure inspiratory and expiratory flow; an analog/digital converter; and, a microcomputer which analyzes signals and computes derived parameters.

For each breath, the computer performed a complete analysis of inspiratory and expiratory gas and displayed the tidal volume (TV), O_2-end and CO_2-end tidal values, oxygen consumption (VO_2 ml/min/m^2), carbon dioxide production (VCO_2 ml/min/m^2), respiratory quotient (RQ). VO_2 and CO_2 were calculated according the conventional formulas; RQ was obtained as VCO_2/VO_2 ratio. Metabolic rate (MR Kcal/m^2/24 hr) was obtained by a previously published computational method.[20]

Statistical Analysis

Data were expressed as the mean ± SD. Statistical analysis was performed by the multiple linear regression technique using Scheffe's test for simultaneous sample variance analysis.[21] The F ratio, which is the ratio of explained to unexplained variability adjusted for the degrees of freedom, and the p value, indicate the reliability of the data.

RESULTS

GLC Analysis of Dicarboxylic Acids

The addition of 50 to 100 μg of DA (C8, C10) as internal standards to both serum and urine samples allowed a recovery of 90% to 98%. The calibration curve showed a linear response of standard derivatized DA with a range of 0.05 to 100 μg; the detection limit was 10 ng. No trace of DA was found in either the serum or the urine samples taken before Az administration. The GLC separation of a synthetic mixture of DA dimethylesters is shown in Figure 1.

Figures 2 and 3 demonstrate the chromatograms of DA present in serum (80 min after starting infusion) and in urine (2 hr after starting infusion) respectively. Serum levels (mean + SD) of Az were measured every 40 min from the start of the infusion until 120 min after the end of the infusion. The serum concentrations of Az peaked at the end of the infusion, with levels over 500 μg/ml. However, at 200 min, only low amounts of Az were detected in serum as shown in Figure 4. In Figures 5 and 6 the urinary excretion curves of azelaic and pimelic acids are given. The excretion of these diacids was highest during the first 2 hr of the study. Both Az and pimelic acid were absent in the samples collected 10 hr after starting the infusion (10–24 hr).

Indirect Calorimetric Data

The oxygen consumption significantly increased during infusion according to the following linear regression:

$$VO_2/m^2 = 121.4 + 0.12 \times \text{time}$$
$$r^2 = 0.14 \quad F\,(1.33) = 6.8$$
$$p < 0.0129 \quad n = 35$$

The carbon dioxide production (VCO_2/m^2) remained constant. Thus the RQ dropped significantly during infusion due to the shift to lipids as the caloric source (Fig. 7). Figure 7 shows the progressive changes of the calories

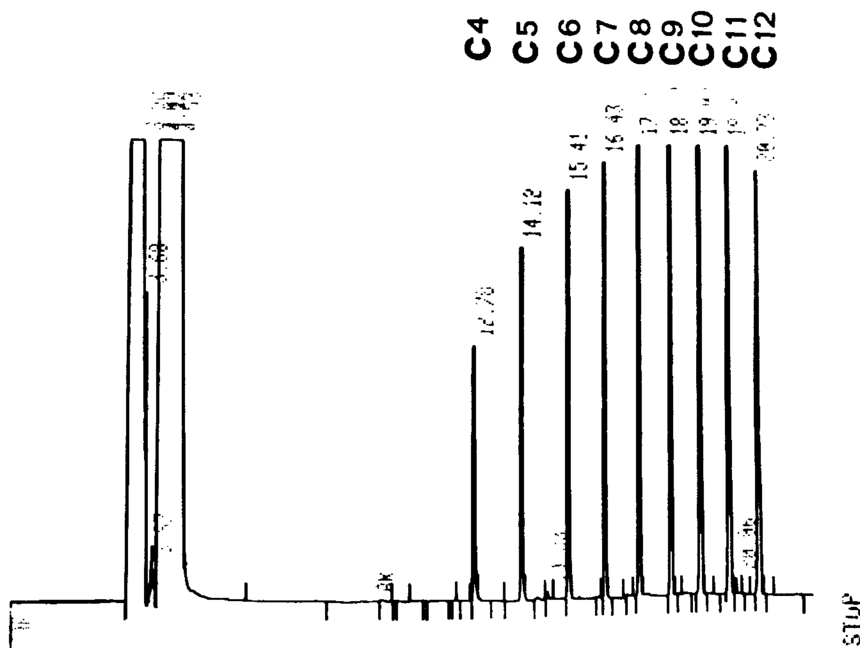


FIG. 1. GLC separation of a mixture of dimetyl esters of standard dicarboxylic acids. Conditions are described in "Materials and Methods." Peak identification: succinic acid (*C4*); glutaric acid (*C5*); adipic acid (*C6*); pimelic acid (*C7*); suberic acid (*C8*); azelaic acid (*C9*); sebacic acid (*C10*); undecanedioic acid (*C11*); dodecanedioic acid (*C12*).

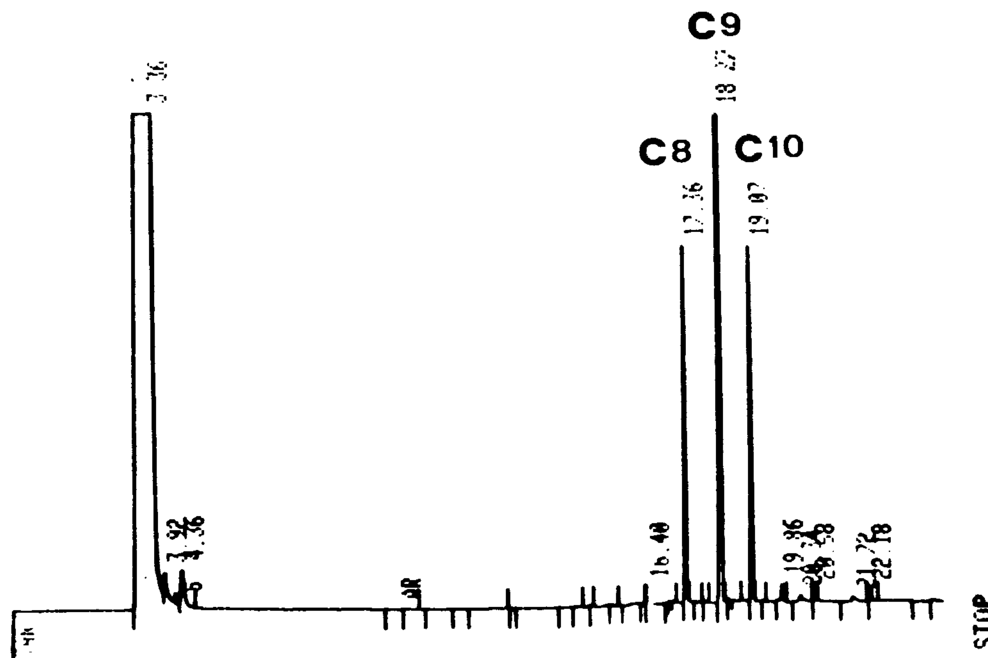


FIG. 2. GLC separation of dimethyl esters of dicarboxylic acids from serum (80 min after starting infusion). Peak identification: see Figure 1.

derived from lipids for each subject examined. Values are expressed as per cent increase greater than baseline values, ie, greater than the amount of calories derived from lipids and measured before Az infusion.

The percentage of calories derived from lipid oxidation increased over basal values according to the equation:

$$\text{Lipid Kal }\% = 0.13 + 0.0055 \times \text{time}$$
$$r^2 = 0.55 \text{ F } (1.33) = 31.9$$
$$p < 0.0001 \text{ n} = 35$$

For further details also see "Appendix of the Results".

No change in urea nitrogen excretion in the urine[22] was observed during the study.

DISCUSSION

Recently, much attention has been focused on the study of alternative substrates in TPN since the fuel use of both iv infused glucose and triglycerides (LCT) appears impaired in some clinical situations.

An impaired glucose tolerance, related to an insulin resistance, has been described in premature newborns, in patients with poorly controlled diabetes mellitus, and

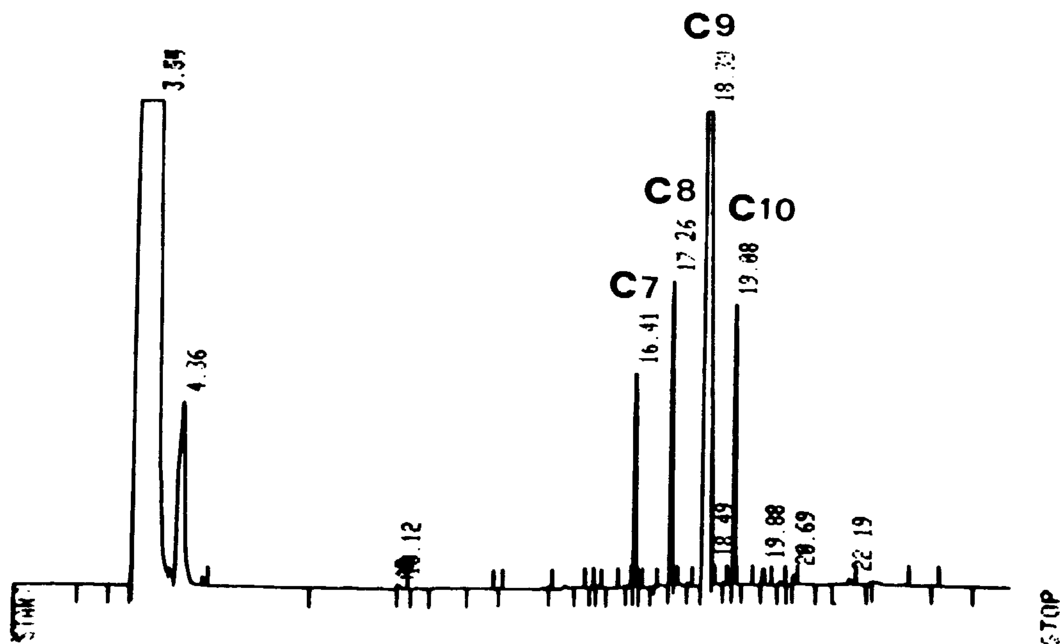


FIG. 3. GLC separation of dimethyl esters of dicarboxylic acids from urine (2 hr after starting infusion) of the same subject. Peak identification: see Figure 1.

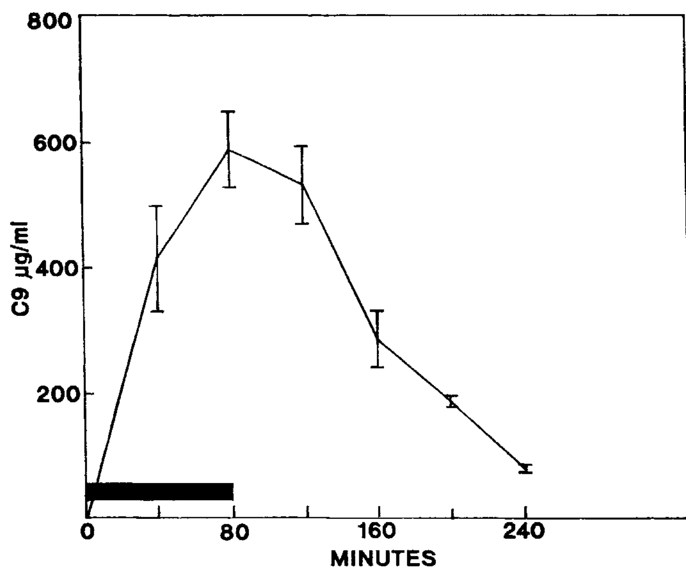


FIG. 4. Serum levels (µg/ml) (mean ± SD) of azelaic acid (AZ) after intravenous infusion of 10 g of the diacid over 80 min (infusion rate = 7.5 g/hr). The *solid line* indicates that Az is infusing during the first 80 min.

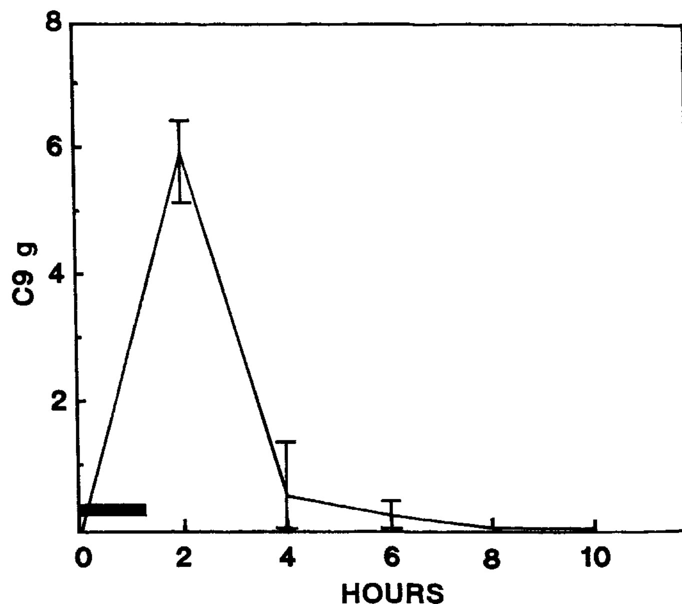


FIG. 5. Urinary excretion curve (mg) (mean ± SD) of azelaic. *Solid line* indicates that Az is infusing during first 80 min.

in critically ill patients.[23] Reduced fuel use of LCT has been found in the late stages of sepsis. It has been suggested that the hypertriglyceridemia may be secondary to a decreased removal of fat from the circulation.[24] Recent studies have confirmed that septic patients have low lipoprotein lipase activity in muscle and adipose tissue.[25] Medium-chain triglycerides were introduced into TPN programs because it was found that the relative carnitine deficiency in sepsis reduces the rate of long-chain fatty acid oxidation in the mitochondria.[25] Studies with animal tissue have shown that medium-chain fatty acids rapidly enter the mitochondria in liver, kidney, and heart, (but not into skeletal muscle) without being converted into acyl carnitine transport forms.[26] In the present work we investigated whether DA could be a suitable alternative substrate in parenteral nutrition.

DA are available in free form and not esterified with glycerol as LCT and MCT. This characteristic is particularly useful in sepsis when the clearance of triglycerides is impaired causing hyperlipidemia[27] and apparently an increased lipid fuel dependency. As in the case of lipid emulsions (LCT or MCT), the iv administration of DA can be performed using a peripheral route and since DA salts are highly soluble in water no emulsions have to be prepared. Also, DA are oxidized in peroxisomes rather than in mitochondria and their intramitochondrial trans-

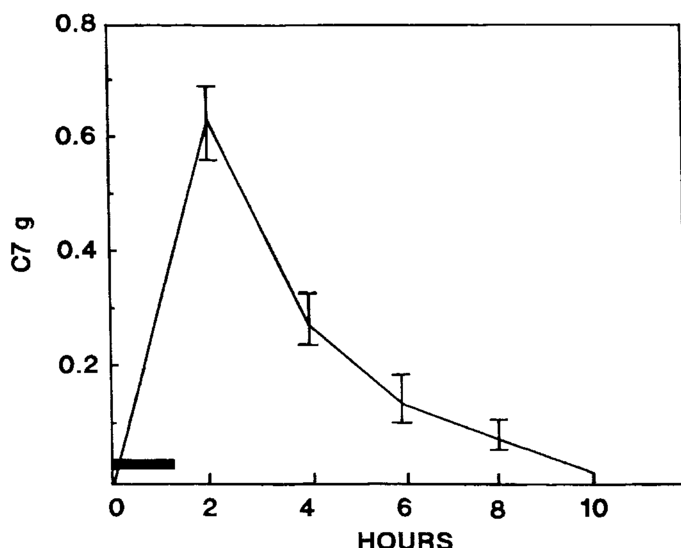


FIG. 6. Urinary excretion curve (mg) (mean ± SD) of pimelic acid. The *solid line* indicates that Az is infusing during the first 80 min.

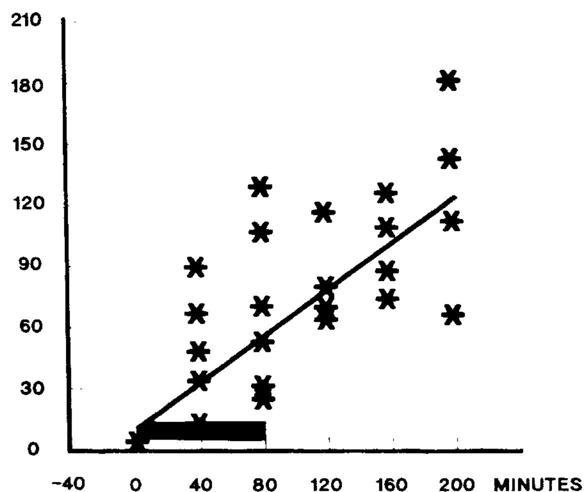
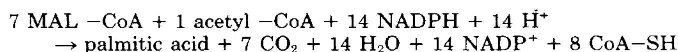


FIG. 7. Change of calories derived from lipid oxidation expressed as per cent increase greater than baseline values.

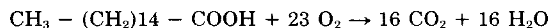
port does not require the presence of carnitine. We chose to use Az instead of other DA because data concerning the acute and chronic toxicity in both animals and humans are available only for Az in the literature. The theoretical basis for the employment of DA in TPN is helpful for understanding its clinical value. The RQ value (0.671) of Az is not only lower than that of glucose (1.00), but also lower than that of long-chain fatty acids (0.69). This is related to the incomplete oxidation of Az, which leads to the production of malonic acid, CO_2 and H_2O as terminal products. Obviously, the absolute CO_2 production from Az is very low (6.38 mM/Kcal). This could represent an advantage in patients with respiratory diseases.

The Kcal/g and ATP/mole ratios of Az are higher than those of glucose (4.97 *vs* 3.70 and 49 *vs* 38, respectively), but lower than for long-chain fatty acids. However, the most interesting data is that the ATP/CO_2 ratio (8.17) is equivalent for both Az and palmitic acid and certainly higher than that for glucose. This indicates that a low production of CO_2 is coupled with a high ATP generation and therefore with the storage of a form of energy necessary for all cellular processes. Therefore, DA could be a suitable substrate for TPN. However, when high doses of Az are administered intravenously there is a great loss in the urine, exceeding 50% of the infused Az. Considering the additional urinary loss of pimelic acid (mean value 1.05 ± 0.16 g), the total amount of Az used in the study period was about 2.5 g. Malonyl-CoA which originates from the β-oxidation of Az is a precursor and the starter of fatty acid synthesis. The synthesis of palmitic acid from acetyl-CoA and malonyl-CoA is described in the following reaction and requires 57.5 ATP:



$$7\ \text{MAL}-\text{CoA} + 1\ \text{acetyl}-\text{CoA} + 14\ \text{NADPH} + 14\ \text{H}^+ \rightarrow \text{palmitic acid} + 7\ CO_2 + 14\ H_2O + 14\ \text{NADP}^+ + 8\ \text{CoA}-\text{SH}$$

Since the β-oxidation of palmitic acid produces 129 ATP, the positive energy balance of the two reactions:



$$CH_3-(CH_2)14-COOH + 23\ O_2 \rightarrow 16\ CO_2 + 16\ H_2O$$

is equivalent to 71.5 ATP. Therefore, if we consider the complete use of the malonyl-CoA derived from Az, the molecules of ATP generated during the intracellular oxidation of Az becomes 59.21, and the energy as Kcal/moles of Az becomes 432.26. Thus the efficiency of the energy balance increases to 36.47%; the Kcal/g are 6.30, ie, an intermediate value between glucose (3.70) and palmitic acid (9.00). However, if Az is completely β-oxidated, the RQ would increase to 0.82 and the ATP/CO_2 ratio would become 6.58:

$$COOH-(CH_2)7-COOH + 11\ O_2 \rightarrow 9\ CO_2 + H_2O$$

The indirect calorimetric study supports the stoichiometric analysis that Az is oxidized *in vivo*. In fact, in all the six subjects examined, O_2 consumption rose 145.4 ml/min/m^2 while the CO_2 production remained constant. On the other hand, the percentage of calories derived from lipid oxidation was greater than the initial value of 120% at the end of Az infusion. This response is demonstrated in Figure 7 by the straight-line function with a slope having a 0.055% Kcal/hr increase.

In conclusion, these preliminary data suggest that DA may serve as a possible substrate in parenteral nutrition. However, the greater urinary loss of the substrate is problematical. This could probably be rectified by a slower infusion rate of Az (we used a rate of 10 g/80 min which is comparable to the lipid emulsion infusion rate currently used in parenteral nutrition) and by modifying the chemical properties of the DA molecule; for example, one of the two terminal carboxylic groups could be made apolar creating an amphypathic molecular structure. Our preliminary studies demonstrated that when the same amounts of sebacic acid (C10) (10 g) are given iv, only about 16% of DA is excreted in the urine. This interesting finding can serve as the basis for further studies of DA as a possible alternative substrate in TPN.

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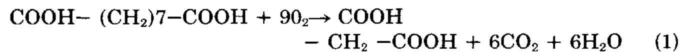
APPENDIX OF THE RESULTS

Stochiometric Analysis of Az Metabolism

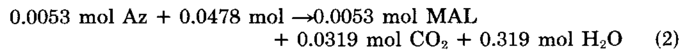
The respiratory quotient (RQ) of Az (MW = 188.2 g) was calculated to be 0.667 using the balanced chemical equation (1) which describes the net biochemical oxidation reaction of this compound:



$$COOH-(CH_2)7-COOH + 90_2 \rightarrow COOH - CH_2 - COOH + 6CO_2 + 6H_2O \quad (1)$$

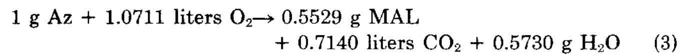
To oxidize 1 g of Az, equivalent to 0.0053 mol, 1.0711

Therefore:



$$0.0053 \text{ mol Az} + 0.0478 \text{ mol} \rightarrow 0.0053 \text{ mol MAL} + 0.0319 \text{ mol } CO_2 + 0.319 \text{ mol } H_2O \quad (2)$$

which corresponds to:



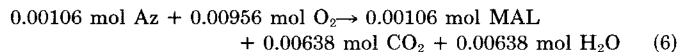
$$1 \text{ g Az} + 1.0711 \text{ liters } O_2 \rightarrow 0.5529 \text{ g MAL} + 0.7140 \text{ liters } CO_2 + 0.5730 \text{ g } H_2O \quad (3)$$

The caloric value per g of Az is 4.9737 Kcal and is derived from equations 4 and 5:

$$3.91. + (1.10 \times RQ) = 3.91 + (1.10 \times 0.667) = 4.6437 \text{ Kcal/liters } O_2 \quad (4)$$

$$1.0711 \times 4.6437 = 4.9738 \text{ Kcal/g Az} \quad (5)$$

Therefore, 1 Kcal of Az is equivalent to 0.00106 mol. The gas exchange associated with the metabolism of 1 Kcal of Az is represented by equation 6:



$$0.00106 \text{ mol Az} + 0.00956 \text{ mol } O_2 \rightarrow 0.00106 \text{ mol MAL} + 0.00638 \text{ mol } CO_2 + 0.00638 \text{ mol } H_2O \quad (6)$$

The comparative amounts of oxygen consumed and carbon dioxide produced per kilocalorie of substrate (glucase, palmitic acid, soybean emulsion and Az) and the correspondent RQ values are reported in Table I.

Since 2 ATP are required for the activation of Az in S-CoA, the β-oxidation of Az up to malonic acid (MAL) produces 49 molecules of ATP.

The standard freen energy, $\Delta G°$, of the hydrolysis of ATP is -7.3 Kcal/mol.[28] The value of $\Delta G°$ and ATP generated during the oxidation of Az are represented in

TABLE I

Respiratory gas exchange per Kcal of substrate derived from oxidation of substrate

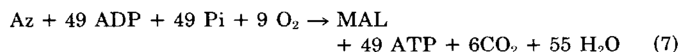
Substrate	Oxidation products	O_2 consumed (mM)	CO_2 produced (mM)	RQ
Glucose	$CO_2 + H_2O$	9.0	9.0	1.0
Palmitic acid	$CO_2 + H_2O$	10.0	6.9	0.7
Soybean emulsion	$CO_2 + H_2O$	10.1	7.1	0.7
Azelaic acid	$MAL + CO_2 + H_2O$	9.5	6.3	0.66

TABLE II
Energetics of fuel metabolism

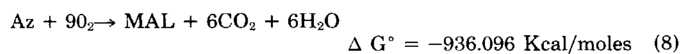
Substrate	Products	Molecular weight	Kcal/g*	ATP mole	ATP/Kcal	ATP/CO_2
Glucose	$CO_2 + H_2O$	180	3.7	38	0.057	6.3
Palmitic acid	$CO_2 + H_2O$	256	9.0	129	0.056	8.1
Soybean emulsion	$CO_2 + H_2O$	271	9.0	138	0.057	7.9
Azelaic acid	MAL + $CO_2 + H_2O$	188	5.0	49	0.052	8.2

* Rounded-off values.

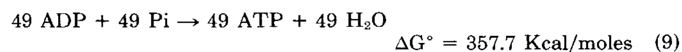
equations 7–11:



$$Az + 49\ ADP + 49\ Pi + 9\ O_2 \rightarrow MAL + 49\ ATP + 6CO_2 + 55\ H_2O \quad (7)$$



$$Az + 9O_2 \rightarrow MAL + 6CO_2 + 6H_2O \quad \Delta G^\circ = -936.096\ Kcal/moles \quad (8)$$



$$49\ ADP + 49\ Pi \rightarrow 49\ ATP + 49\ H_2O \quad \Delta G^\circ = 357.7\ Kcal/moles \quad (9)$$

$$\text{Efficiency of energy recovery} = 358 \times 100/936 = 38.28\% \quad (10)$$

$$ATP/CO_2\ \text{molar ratio} = 49/6 = 8.17. \quad (11)$$

The values of ΔG°, efficiency of energy recovery, and ATP/CO_2 molar ratio of glucose, palmitic acid, and Az, respectively, are reported in Table II.

One thousand Kcal of Az produce 143.57 liters of CO_2 as seen in equation 12:

$$1000\ Kcal\ Az \rightarrow 0.064\ mol\ CO_2/Kcal \times 22.4\ liters/mol \times 1000\ Kcal = 143.575\ liters\ CO_2 \quad (12)$$

This can be compared to Silberman and Silberman's[29] calculation for the oxidation of 1000 Kcal of glucose and 1000 Kcal of soybean emulsion which produced 202 and 157 liters of CO_2, respectively.