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Metabolism of an ionic contrast medium and the related agents

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Abstract

Liquid chromatography–atmospheric pressure chemical ionization mass spectrometry was applied to analyze the iodinated compounds and their glycine conjugates. The negative-ion mass spectra of the iodinated compounds gave $[M-H]^-$, $[M-COOH]^-$ and $[I]^-$ ions. The positive- and negative-ion mass spectra of the glycine conjugates showed abundant $[M+H]^+$ and $[M-H]^-$ ions. Fragmentations of the glycine conjugates obtained in the positive-ion mode were different from those in the negative-ion mode, the former providing more useful structural information for the presence of glycine. Mouse kidney mitochondria were more active in glycine conjugation than liver mitochondria. Mono-substituted benzoic acids were conjugated with glycine in liver and kidney, whereas the acids having three functional groups or more did not undergo glycine conjugation in liver and kidney.

Keywords: Benzoic acid; Glycine conjugates

1. Introduction

There are many reports about the adverse effects with the increased number of radiographic examinations [1–3]. The adverse reaction consists of anaphylactic shocks, hypotension, allergy, skin rash, pulmonary edema, etc. In addition, the delayed adverse reaction due to the ionic and nonionic contrast media occasionally occurs. However, the mechanism of the adverse effects remains unclear.

The ionic contrast media are benzoic acid derivatives, which generally undergo conjugation with glycine. Glycine conjugation is the most important route of detoxification. Glycine conjugation proceeds through a two-step reaction. The carboxylic acid is first converted to a high-energy CoA thioester by medium chain acyl-CoA synthetases. The activated acid is then transferred from the CoA to the amino group of glycine in a reaction catalyzed by acyl-CoA:glycine *N*-acyltranferases. Xenobiotic acyl-CoA species, which are formed at the first step in glycine conjugation, may occasionally serve as precursors in the formation of hybrid triacylglycerols. Then, xenobiotics may be accumulated in tissue. The effect of the ionic contrast media on lipid-biosynthetic processes and the metabolism of fatty acids may result in causing the delayed adverse reaction.

Ionic contrast media are chemically considered to be stable. However, Diatrizoate (3,5-diacetamido-2,4,6-triiodobenzoic acid) was reported to be transformed to reductive deiodinated compounds [4]. Therefore, it is necessary to examine the metabolic fate of deiodinated compounds. To elucidate the mechanism of the toxicity induced by the ionic contrast media, the iodinated compounds synthesized

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during the development of radiodiagnostic agents were also selected [5].

We investigated the extent to which an ionic contrast medium and the related compounds were conjugated with glycine. We also analyzed a series of iodinated compounds and their glycine conjugates by using liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS). In addition, we predicted whether a class of acyl-CoAs implicated in toxicity is formed or not.

2. Experimental

2.1. Chemicals

Benzoic acid, 2-amino-, 2-iodo-, 3-iodo- and 4-iodo-benzoic acids were purchased from Nacalai Tesque (Kyoto, Japan). 2-Amino-3,5-diiodo-, 4-amino-3,5-diiodo-, 2-hydroxy-3,5-diiodo-, 2,3,5-triiodo- and 3-amino-2,4,6-triiodo-benzoic acids were obtained from Aldrich (Milwaukee, WI, USA). 3-Acetylamino-2,4,6-triiodo- and 3,5-diacetylamino-2,4,6-triiodo-benzoic acids were from Sigma. All other chemicals were of reagent grade.

2.2. Assay of glycine conjugation

The animals used were ddY strain male mice weighing about 25–30 g. Formation of glycine conjugates in mouse liver and kidney mitochondria was assayed as described previously [6,7].

Briefly, the liver and kidney were washed with a cold 0.25 *M* sucrose–10 m*M* Tris–HCl buffer (pH 7.4) and homogenized with four volumes of the same buffer, respectively. Homogenates were centrifuged at 700 *g* for 15 min. The resulting supernatant was then centrifuged at 9700 *g* for 20 min. The mitochondrial pellet obtained was resuspended in 0.2 *M* Tris–HCl buffer (pH 8.5). Incubation mixtures (2.60 ml) consisted of each iodinated compound (0.3 μmol), ATP (15 μmol), glycine (60 μmol), $MgCl_2$ (3 μmol) and intact mitochondria in 0.2 *M* Tris–HCl buffer (pH 8.5). After incubation for 1 h at 37°C, the reaction was stopped by the addition of 0.35 ml of 7 *M* HCl. After centrifugation, 0.35 ml of 7 *M* NaOH was added to the supernatant. Aliquots of the neutralized supernatant were subjected to high-performance liquid chromatography (HPLC) analysis. Protein contents (corresponding to 6–8 mg protein for liver and 3–6 mg protein for kidney) were used so that about 55–65% and 85–95% of benzoic acid for the liver and kidney were conjugated with glycine in 1 h, respectively.

It was confirmed that the decreasing peak of the substrate resulted in the concomitant increasing peak of the glycine conjugate on the chromatogram. Therefore, the rate of each glycine conjugate was calculated as the decreasing rate of the substrate.

2.3. Protein analysis

Protein was determined in duplicate at 595 nm with protein assay kit (Bio-Rad).

2.4. Purification of a medium chain acyl-CoA synthetase

The medium chain acyl-CoA synthetase was purified from mouse kidney as described previously [8,9].

2.5. Coupled enzyme assay of the medium chain acyl-CoA synthetase

The activity of the medium chain acyl-CoA synthetase was determined as described previously [8].

Briefly, medium chain acyl-CoA synthetase activities with various iodinated acids were determined by coupling the formation of AMP to myokinase, pyruvate kinase and lactate dehydrogenase, and monitoring the oxidation of NADH at 340 nm. The reaction mixture consisted of each iodinated acid (1 μmol), NADH (0.36 μmol), CoA (0.6 μmol), ATP (3 μmol), $MgCl_2$ (20 μmol), phosphoenolpyruvate (1 μmol), KCl (29 μmol), myokinase (2 unit), pyruvate kinase (2 unit), lactate dehydrogenase (2 unit) and the medium chain acyl-CoA synthetase in a total volume of 2.0 ml of 0.2 *M* Tris–HCl buffer (pH 8.5). After incubation at 37°C for 30 min, absorbance of the mixture was monitored at 340 nm.

Table 1
Mobile phases for separation of the iodinated compounds and their glycine conjugates

Mobile phase, water–methanol–acetonitrile–acetic acid	The ionic contrast medium and the related reagents
(a) 30:35:35:0.6 (v/v)	2-Hydroxy-3,5-diiodobenzoic acid
(b) 44:28:28:0.6 (v/v)	2-Amino-3,5-diiodobenzoic acid, 2,3,5-Triiodobenzoic acid
(c) 50:25:25:0.6 (v/v)	3-Iodobenzoic acid, 4-Iodobenzoic acid, 3-Amino-2,4,6-triiodobenzoic acid, 4-Amino-3,5-diiodobenzoic acid
(d) 60:20:20:0.6 (v/v)	Benzoic acid, 2-iodobenzoic acid
(e) 80:10:10:0.6 (v/v)	3-Acetylamino-2,4,6-triiodobenzoic acid, 2-Aminobenzoic acid
(f) 84:8:8:0.6 (v/v)	3,5-Diacetylamino-2,4,6-triiodobenzoic acid

2.6. Instrumentation

HPLC separation was performed with a Hitachi (Tokyo, Japan) L-6200 instrument equipped with a 5 μm Cosmosil-MS C_{18} column (150 mm×4.5 mm I.D.) (Nacalai Tesque). The mobile phases were water–methanol–acetonitrile–acetic acid systems as follows: (a) 30:35:35:0.6 (v/v); (b) 44:28:28:0.6 (v/v); (c) 50:25:25:0.6 (v/v); (d) 60:20:20:0.6 (v/v); (e) 80:10:10:0.6 (v/v), (f) 84:8:8:0.6 (v/v). The flow-rate was 1 ml/min. The mobile phases used for separation of the iodinated compounds and their glycine conjugates are given in Table 1. The column effluent of each acid and its glycine conjugate was monitored at 240 nm.

The positive- and negative-ion mass spectra were measured with a Hitachi M-2000 double-focusing mass spectrometer equipped with an atmospheric pressure ionization source. All mass spectral data were obtained by scanning the mass range from m/z 1 to 700 in 4 s, with dwell time of 0.5 s. The nebulizer temperature was set at 300 or 350°C. The drift voltage varied in the range 60–85 V.

3. Results and discussion

For direct simultaneous determination of each iodinated acid and its glycine conjugate, we investigated the specific, simple and sensitive procedure by HPLC. Further, to identify the glycine conjugates of the iodinated compounds formed in mouse liver and kidney mitochondria, we applied LC–APCI-MS. Because of the very different lipophilicity of the iodinated acids, the analysis had to be carried out by using six mobile phases. Fig. 1 shows typical extracted ion profiles of the extracts obtained after incubation with (A) 2-iodobenzoic acid and (B) 3-iodobenzoic acid in mouse kidney mitochondria.

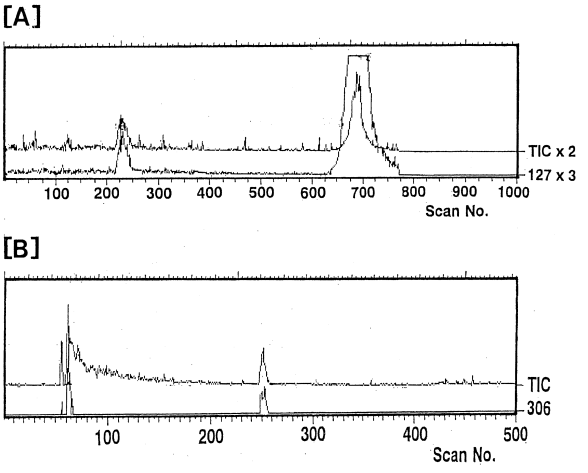

Fig. 1. Typical extracted ion profiles of the extracts obtained after incubation with (A) 2-iodobenzoic acid and (B) 3-iodobenzoic acid in mouse kidney mitochondria.

Individual peaks were monitored by (A) the $[I]^-$ ion and (B) the $[M+H]^+$ ion of the acids or their glycine conjugates. Each increasing peak was identified as the glycine conjugate from the mass spectral fragmentation patterns which were given as under. An acid and its glycine conjugate were well separated and could be determined simultaneously.

It is necessary for identification of the metabolites to obtain structural information. Therefore, the optimum analytical conditions were investigated to obtain some structural information. The positive- and negative-ion mass spectra of the ionic contrast medium and the related compounds were measured using water–methanol–acetic acid (50:50:0.6, v/v) as the mobile phase at a flow-rate of 1.0 ml/min in the flow-injection mode (no column). Benzoic acid reacts as a strong acid in the gas phase, resulting in the generation of negative ions in the APCI source. Therefore, the mass spectra of the ionic contrast medium and the related compounds could be achieved in the negative-ion mode. In the case of a low drift voltage, the negative-ion mass spectra of mono-substituted benzoic acids gave dominant $[M-H]^-$ ions with low-abundance cluster ions, whereas the negative-ion mass spectra of other iodinated compounds did not show dominant $[M-H]^-$ ions with the increased number of the substituent on the benzene ring. To obtain structural information, the drift voltage was kept at 80 V.

Figs. 2 and 3 show the negative-ion mass spectra of benzoic acid, 2-iodo-, 3-iodo-, 4-iodo-, 2-amino-3,5-diiodo-, 4-amino-3,5-diiodo-, 2-hydroxy-3,5-diiodo-, 2,3,5-triiodo-, 3-amino-2,4,6-triiodo-, 3-acetylamino-2,4,6-triiodo- and 3,5-diacetylamino-2,4,6-triiodo-benzoic acids. The negative-ion mass spectra of the iodinated compounds generally gave $[M-H]^-$ and $[M-COOH]^-$ ions. The ion at m/z 127 indicated the presence of iodo moiety.

To obtain characteristic fragmentations of the glycine conjugates, the drift voltage was kept at 85 V. Fig. 4 shows the negative-ion mass spectra of the glycine conjugates of 2-iodo-, 3-iodo- and 4-iodo-benzoic acids. All the negative-ion mass spectra gave $[M-H]^-$ at m/z 304, $[M-COOH]^-$ at m/z 260 and $[I]^-$ at m/z 127. This fragmentation was the same as with the iodinated benzoic acids. Only the $[M-CONH_2COOH]^-$ ion was observed as the characteristic ion for the glycine conjugates.

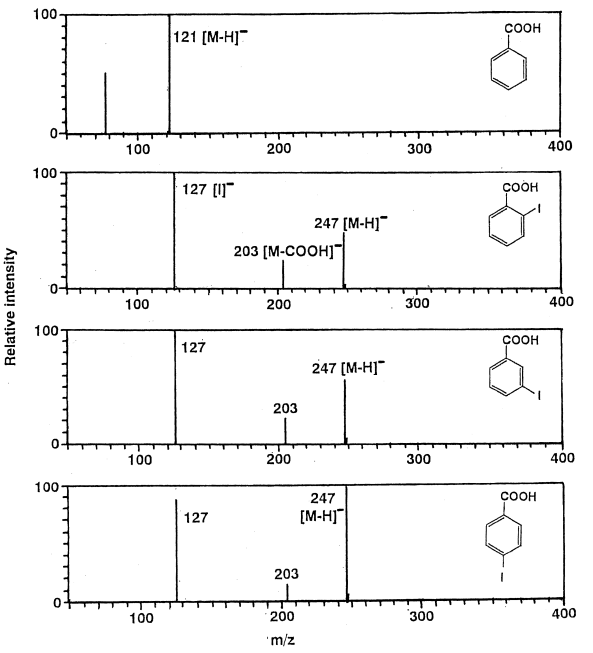

Fig. 2. The negative-ion mass spectra of benzoic acid, 2-iodo-, 3-iodo- and 4-iodo-benzoic acids.

Fig. 5 shows the positive-ion mass spectra of the glycine conjugates of 2-iodo-, 3-iodo- and 4-iodo-benzoic acids. The mass spectra of the glycine conjugates of the iodinated compounds gave $[M+H]^+$ at m/z 306, $[M+Na]^+$ at m/z 328, $[M-CH_2COOH+2H]^+$ at m/z 248 and $[M-NHCH_2COOH]^+$ at m/z 231. The positive-ion mass spectra of the glycine conjugates were different from their negative-ion mass spectra, the former providing more useful structural information for the presence of glycine. The glycine conjugates of mono-iodinated acids could be identified in mouse liver and kidney mitochondria.

Fig. 6 shows glycine conjugation of the iodinated compounds in mouse liver and kidney mitochondria. About 80–100% of benzoic acid was conjugated with glycine in a variety of animals. To compare with the iodinated compounds, benzoic acid was also selected. There were the differences in glycine conjugation of mono-substituted benzoic acids between liver and kidney mitochondria. Mouse kidney mitochondria were more active in glycine conjugation than liver mitochondria. 4-Iodobenzoic acid showed a high degree of glycine conjugation in liver

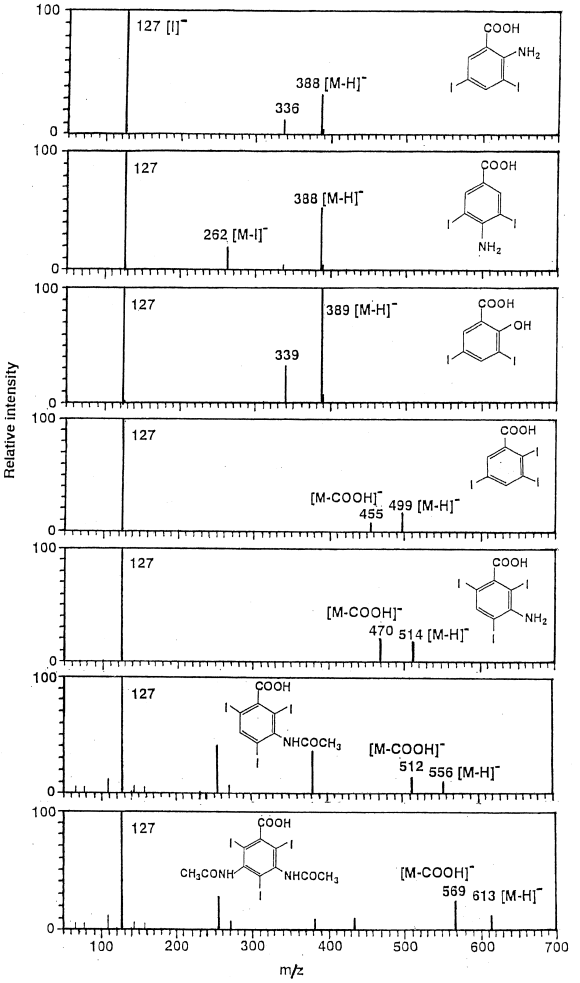

Fig. 3. The negative-ion mass spectra of 2-amino-3,5-diiodo-, 4-amino-3,5-diiodo-, 2-hydroxy-3,5-diiodo-, 2,3,5-triiodo-, 3-amino-2,4,6-triiodo-, 3-acetylamino-2,4,6-triiodo- and 3,5-diacetylamino-2,4,6-triiodo-benzoic acids.

and kidney. The extent of glycine conjugation of 3-iodobenzoic acid was six times greater in kidney than in liver. However, 2-iodo- and 2-amino-benzoic acids were conjugated to a small extent with glycine in liver and kidney. We have previously reported that the conjugation of salicylic acid with glycine took place in only the mouse kidney mitochondria [9]. *Ortho*-substituted acids showed a low degree of glycine conjugation. The acids having three functional groups or more did not undergo glycine conjugation in liver and kidney. The lipophilicity of compounds became greater with the increased number of the iodo group. However, they were not conjugated with glycine.

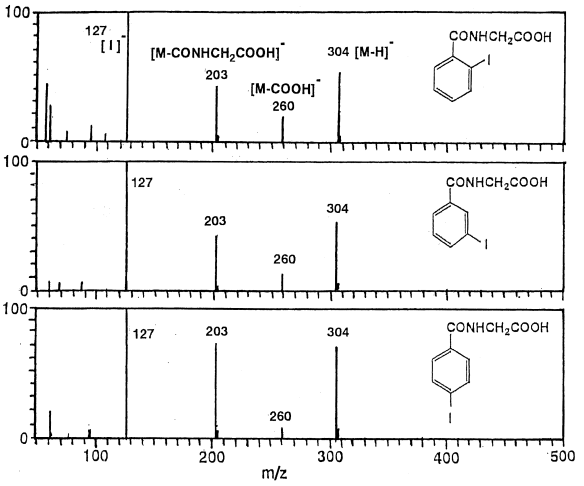

Fig. 4. The negative-ion mass spectra of the glycine conjugates of 2-iodo-, 3-iodo- and 4-iodo-benzoic acids.

The toxicity in terms of the lethal dose giving 50% deaths (LD_{50}) (in mouse) for the iodinated compounds has been reported by Knoefel and Haung [5]; benzoic acid (LD_{50}=16 m*M*/kg), 2-iodobenzoic acid (3.2 m*M*/kg), 3-iodobenzoic acid (2.8 m*M*/kg), 4-iodobenzoic acid (2.0 m*M*/kg), 2-hydroxy-3,5-diiodobenzoic acid (0.40 m*M*/kg), 2-amino-3,5-diiodobenzoic acid (0.90 m*M*/kg), 4-amino-3,5-

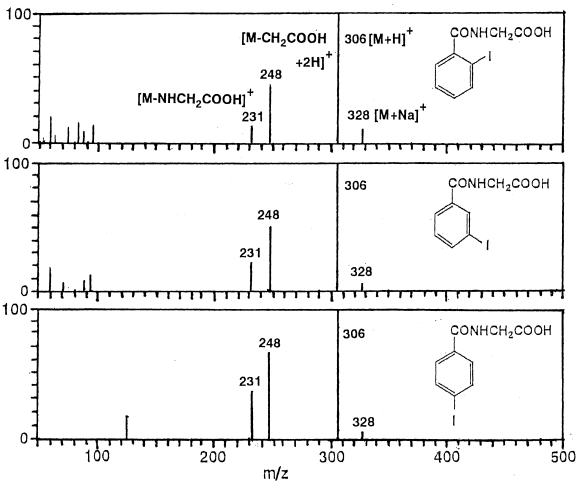

Fig. 5. The positive-ion mass spectra of the glycine conjugates of 2-iodo-, 3-iodo- and 4-iodo-benzoic acids.

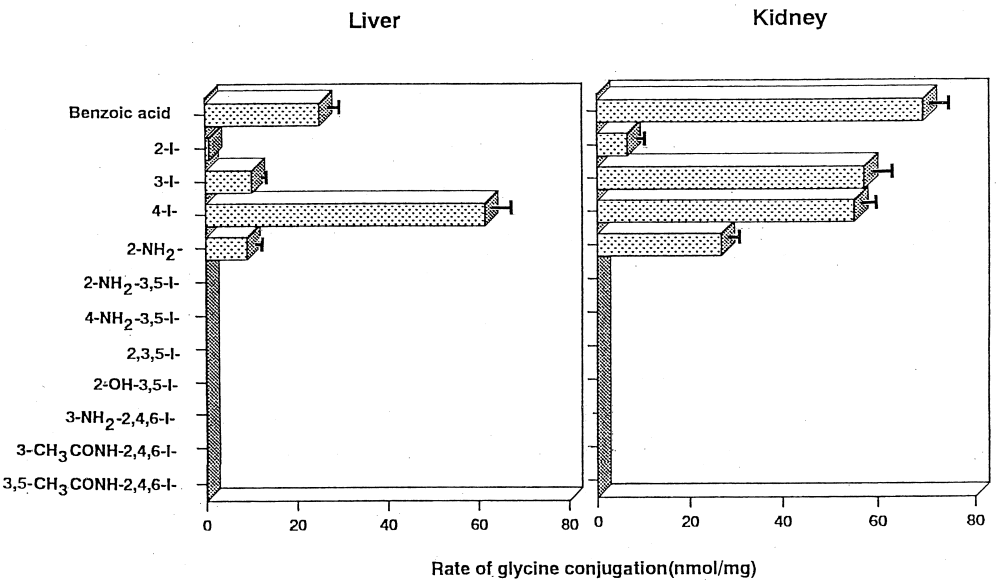

Fig. 6. Glycine conjugation of the iodinated compounds in mouse liver and kidney mitochondria. Values represent the average of four experiments.

diiodobenzoic acid (1.4 m*M*/kg), 2,3,5-triiodobenzoic acid (0.30 m*M*/kg), 3-amino-2,4,6-triiodobenzoic acid (2.9 m*M*/kg), 3-acetylamino-2,4,6-triiodobenzoic acid (21 m*M*/kg), 3,5-diacetylamino-2,4,6-triiodobenzoic acid (32 m*M*/kg). The increase in toxicity followed the substitution of one iodine, when the only iodine atoms are the substituent. Introduction of the hydroxyl or amino group in the *ortho*-position resulted in an increase in toxicity. The addition of the acetylamino group was associated with highly significant reduction in toxicity. When all the positions on the ring were occupied, the lowest toxicity was observed. To elucidate the determinants implicated in toxicity, we investigated the relationship between LD_{50} values and glycine conjugation of iodinated compounds in liver and kidney mitochondria. There were no correlations ($r=0.285$ for liver and $r=0.662$ for kidney) between glycine conjugation and LD_{50} values. It appears that the toxicity does not reduce with an increase in formation of glycine conjugation. However, glucuronic acid conjugation is another route of detoxification of xenobiotic carboxylic acids. Therefore, it may be also necessary to investigate glucuronic acid conjugation of the iodinated compounds.

The first reaction of glycine conjugation involves the active intermediates (acyl-CoAs), which may occasionally be precursors in the formation of hybrid triacylglycerols. Then, xenobiotics may be accumulated in the liver, adipose and skin. This pathway includes various interactions with lipid-biosynthetic or metabolic processes. Therefore, we examined whether the iodinated compounds serve as the substrates for the medium chain acyl-CoA synthetase catalyzing the first reaction of glycine conjugation or not. Little information is available about the medium chain acyl-CoA synthetases. We previously purified one medium chain acyl-CoA synthetase [8,9]. One purified medium chain acyl-CoA synthetase was shown to accept not only medium chain fatty acids but also aromatic acids. The highest activity was found with hexanoic acid. The enzyme activities were observed with benzoic acid, 3-iodo-, 4-iodo- and 4-amino-3,5-diiodo-benzoic acids (Fig. 7). However, *ortho*-substituted acids exhibited no activity. The acids with three groups or more did not serve as substrates. Another was reported to be the salicylate activating enzyme, which had been partially purified over 30-fold by Killenberg et al. [10]. Further, it will be necessary to investigate whether the iodinated compounds are the substrates for the salicylate activating enzyme. Since the iodinated compounds

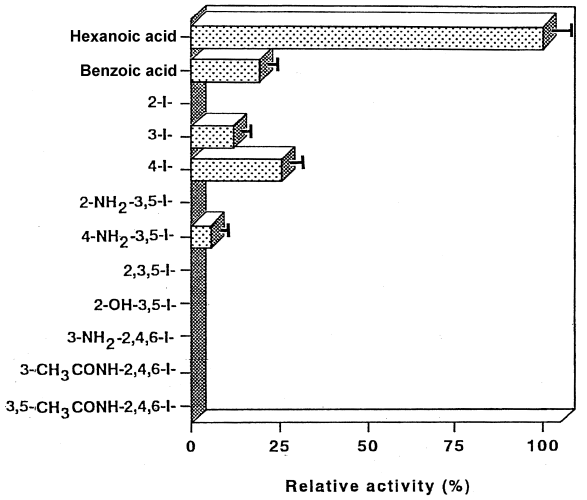

Fig. 7. Substrate specificity of the medium chain acyl-CoA synthetase from mouse kidney mitochondria. Activities are expressed as percent of the specific activity of hexanoic acid. The specific activity for hexanoic acid was 510 nmol/min/mg protein.

were not conjugated with glycine, they may not serve as substrates for the salicylate-activating enzyme. These findings indicate that a large volume of the ionic contrast medium is not competitive with respect to medium chain fatty acids including hexanoic acid and does not disturb the metabolism of lipids. It is also suggested that the ionic contrast medium would not be incorporated into triacylglycerols.

In conclusion, LC–APCI-MS was applied to identify the glycine conjugates of the iodinated compounds. The negative-ion mass spectra of the iodinated compounds gave $[M-H]^-$, $[M-COOH]^-$ and I^- ions. The negative- and positive-ion mass spectra of the glycine conjugates revealed the presence of glycine and an iodo group from the characteristic fragmentation patterns. The benzoic acids having three functional groups or more were not conjugated with glycine.

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