HEREDITARY TYROSINEMIA

Formation of Succinylacetone–Amino Acid Adducts

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Hereditary tyrosinemia is an inborn error of tyrosine metabolism due to an inherited deficiency of fumarylacetoacetate hydrolase activity transmitted in an autosomal recessive fashion (1). This disease is characterized by multiple renal tubular defects accompanied with aminoaciduria and proteinuria, nodular cirrhosis, vitamin D–resistant (hypophosphatemic) rickets, and occasionally symptoms resembling those of acute intermittent porphyria (AIP)[1] (1, 2). Hepatoma is a quite frequent complication ~40% of patients suffering ultimate liver failure (3). Patients with this hereditary disease excrete large amounts of 4,6-dioxoheptanoic acid (succinylacetone [SA]) (4), and the presence of SA is thought to be a specific biochemical marker of this disease. SA in biological fluids can be determined by gas chromatography (4, 5) or indirectly by inhibition of δ-aminolevulinic acid (ALA) dehydratase, because the compound is an extremely potent inhibitor of this enzyme in human as well as in animal tissues (6, 7).

During the course of our studies of tyrosinemia, we observed that urines of patients with hereditary tyrosinemia contain a material(s) that absorbs light at 315 nm. The 315 nm material occurs in urines from patients with tyrosinemia, but not in normal controls, nor in patients with other forms of aminoaciduria or proteinuria. This phenomenon led us to examine the nature of the 315 nm material in detail. We report in this paper that SA reacts nonenzymatically with various amino acids and proteins such as albumin at physiological pH and temperature, and forms stable adducts that have an absorption maximum at 315 nm. High-performance liquid chromatography (HPLC) and thin layer chromatography (TLC) analyses of urines demonstrated that a large amount of SA adducts with amino acids and protein (presumably albumin) are excreted into the urine of patients with hereditary tyrosinemia, but not in control subjects or in patients with other disorders.

Materials and Methods

Materials. SA was purchased from Calbiochem-Behring Corp. (San Diego, CA). Amino acids, reduced glutathione (GSH), and human serum albumin (fraction V) were obtained from Sigma Chemical Co. (St. Louis, MO). Proteinase K was from Boehringer Mannheim Biochemicals (Mannheim, Federal Republic of Germany). All other chemicals were of

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[1] *Abbreviations used in this paper:* ALA, δ-aminolevulinic acid; AIP, acute intermittent porphyria; GSH, reduced glutathione; HPLC, high-performance liquid chromatography; SA, succinylacetone; TLC, thin-layer chromatography; UV, ultraviolet.

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analytical grade. [^{14}C(U)]lysine ([^{14}C]Lys) (324.7 mCi/m mol) was obtained from New England Nuclear (Boston, MA).

Urine samples of three patients with hereditary tyrosinemia, two patients with Fanconi syndrome and two patients with nephrotic syndrome were obtained from the New York Hospital–Cornell University Medical Center (New York), and the Children's Hospital Medical Center–Harvard Medical School (Boston, MA), and urine samples of six normal subjects and two patients with AIP were obtained from The Rockefeller University Hospital. All urine samples were stored at −20°C until use.

Spectrophotometric Analyses of SA–Amino Acid Adducts. Urine samples were diluted 50-fold with distilled water before determination. SA (1 mM) was dissolved in 50 mM sodium phosphate buffer at various pH. After confirming the pH of the final solution, the absorbance spectrum of SA was examined. To prepare the SA–amino acid adduct, SA and amino acids were dissolved in 50 mM sodium phosphate buffer, pH 7.4, and incubated at 37°C. Absorbance spectra were obtained at intervals using the nonincubated solution as the blank. Spectrophotometric measurements of urine samples were performed on a CARY 118 spectrophotometer (Varian Associates, Inc., Palo Alto, CA).

To determine the reactivity of amino acids with SA, the increment of absorbance at 315 nm with time was recorded. Effects of pH on the stabilities of SA–amino acid adducts that had or had not been reduced with sodium borohydride were examined by recording the absorbance spectrum at various pH between 1.0 and 9.0.

Preparation of SA-Albumin Adducts. Human serum albumin was incubated with or without 25 mM SA in 50 mM sodium phosphate buffer, pH 7.4, for 10 min, or for 24 h at 37°C. Final concentrations of SA and albumin were 25 mM and 50 mg/ml (or 750 μM), respectively. The mixture was then dialyzed overnight using a Spectrapor dialysis membrane (cutoff, 6,000–8,000 mol wt) at 4°C against 50 mM phosphate buffer, pH 7.4. The dialyzed albumin and SA-albumin were filtered through a Millipore filter (0.45 μm) (Bedford, MA). Absorbance of the filtrate was determined spectrophotometrically. Protein concentrations were determined by the method of Lowry et al. (8). In some experiments, SA-albumin was reduced with $NaBH_4$ before digestion with proteinase K as described below.

Enzymatic Digestion. Albumin and SA-albumin (5 mg protein) were digested with an equal volume of the digestion buffer (0.1 M Tris-HCl, pH 8.8 containing 1 mM EDTA and 1 mM dithiothreitol) and 1 mg/ml proteinase K (9). The mixture was then incubated at 37°C. Proteolysis was determined to be complete in 2 h, as judged by the generation of ninhydrin-positive material (10). After digestion, extraction of SA-adducts that had been released from proteins was carried out as described below.

For enzymatic digestion of proteins in urine, 20 μl of a mixture containing 500 mM EDTA, pH 8.0, and proteinase K (final concentration, 0.5 mg/ml) were added to a 10-ml urine sample (pH 7.4) that had been diluted with an equal volume of distilled water. Digestion was carried out at 37°C for 2 h.

Extraction of SA and SA–Amino Acid Adducts from Urine. To determine the rate of the extraction of SA and SA adducts in urine, SA or SA-Lys was added to 10 ml normal urine that had been adjusted to pH 7.4 (final concentration, 5 mM as SA), with or without proteinase K, and extracted at pH 7.4, once with two volumes of ethylether and once with two volumes of chloroform/methanol (2:1 vol/vol), and dried under nitrogen stream. The extracts were dissolved in 50% methanol in water, and filtered through a Millex-HV_4 filter (Millipore) for HPLC analysis.

HPLC Analyses. HPLC analyses were carried out on a μBondapack C_{18} column (300 × 4 mm; Waters Associates, Milford, MA) using a Series 3 liquid chromatograph (Perkin-Elmer Corp., Norwalk, CT) equipped with a LC-75 spectrophotometric detector (Perkin-Elmer Corp.). The solvent systems used were as follows: the starting eluent, 20 mM potassium phosphate buffer, pH 7.0, the limiting eluent, methanol/water (1:1 vol/vol), linear gradient elution, from 0 to 100% of the limiting eluent in the starting eluent over a course of 30 min. A flow rate of 1.0 ml/min was used. Absorbance was determined at 315 nm for SA–amino acid adducts, 280 nm for SA and 210 nm for Lys, respectively.

TLC Analyses. We added 1 ml of SA (100 mg) to 0.5 ml of 10 mM Lys containing 10

μCi of [^{14}C]Lys, and incubated for 24 h at 37°C. The mixture was reduced with $NaBH_4$ and was spotted on a silica gel 60 plate (E. M. Merck, Darmstedt, Federal Republic of Germany). Compounds were visualized by iodide vapor, and then autoradiographed. Exposure was performed with Kodak X-OMATAR5 X-ray film (Eastman Kodak Co., Rochester, NY) for 7 d. Urine samples, and SA-albumin that had been digested with proteinase K and then reduced with $NaBH_4$, were extracted with ethylether, followed by chloroform/methanol (2:1 vol/vol). Dried materials were dissolved in 50% methanol in water, and spotted on a silica gel 60 plate. The solvent used for separation was a mixture of isopropanol in 5% ammonium hydroxide (4:1 vol/vol).

Results

Absorbance Spectra of Urine Samples. All urine samples from three patients with hereditary tyrosinemia showed an absorption maximum at 315 nm. Representative absorption spectra from a patient with tyrosinemia are shown in Fig. 1*A*. The peak at 315 nm in the absorbance spectrum was increased when the pH was elevated to 7.4. When SA-Lys was added to the urine sample, it increased the absorbance at 315 nm. In contrast, urine samples from six normal subjects and from six patients with other diseases, including AIP, nephrotic syndrome, and Fanconi syndrome, did not show an absorption peak at 315 nm (Fig. 1*B*). Addition of SA (100 μM) to normal urine did not change the spectrum. A 315

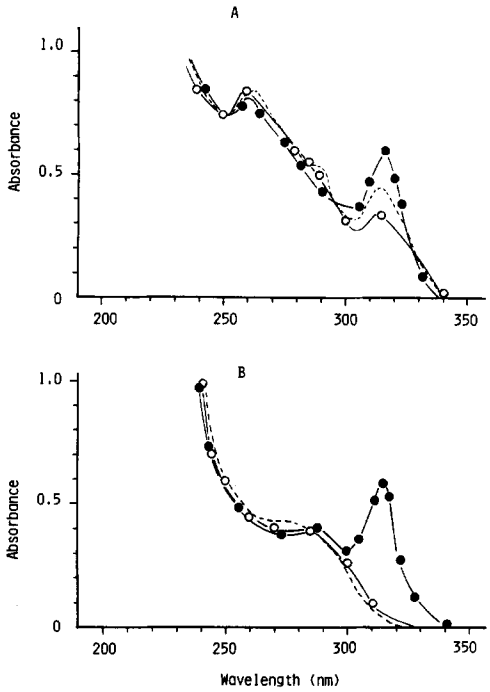

FIGURE 1. Ultraviolet (UV) absorbance spectra of urine samples from a normal subject and a patient with hereditary tyrosinemia. *A*, (○) Urine from a patient with hereditary tyrosinemia diluted 50-fold with distilled water; (– – –) after incubation of the diluted urine sample at 37°C, for 30 min at pH 7.4; (●) after addition of SA-Lys (20 μM), to the diluted urine. *B*, (○) Urine from a normal subject diluted 50-fold with distilled water; (– – –) after addition of SA (100 μM, final concentration) to the diluted normal urine at pH 7.4; (●) after addition of SA-Lys (50 μM) to the diluted normal urine.

nm absorbance material, however, could be readily demonstrated in normal urine when it was incubated with SA-Lys (50 μM) (Fig. 1*B*). In addition to the 315 nm peak, tyrosinemia urines contained material(s) with an absorption peak at 260 nm, which most likely reflects the high phenylalanine content of these urine samples.

Absorbance Spectra of SA and SA–Amino Acid Adducts. SA has an absorbance peak at 280 nm at pH 7.4, while Lys has no absorption at this wavelength (Fig. 2). When UV absorbance spectra of the mixture were recorded using a nonincubated mixture of SA and Lys as reference, a new absorption maximum similar to Fig. 1 appeared at 315 nm, indicating that SA nonenzymatically formed an adduct with Lys.

Relationships between absorbance and concentrations of SA and SA-Lys were examined (Fig. 3). SA and SA-Lys were determined at pH 7.4 at their absorption maxima, i.e., at 280 and 315 nm, respectively (Fig. 3). The concentrations of SA-Lys adducts were expressed as SA equivalents. There was a linear relationship between the concentration and the absorbance of SA and SA-Lys (Fig. 3). Minimal detection limits, i.e., an absorbance of 0.02, were 12.8 μM for SA and 1.3 μM for SA-Lys, respectively.

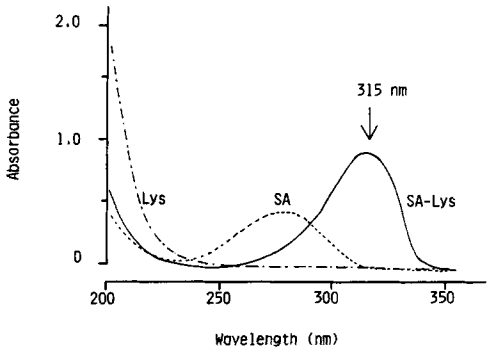

FIGURE 2. UV absorbance spectra of Lys, SA, and SA-Lys. Concentrations of Lys, SA, and SA-Lys were 0.3 mM, 0.3 mM and 0.1 mM, respectively.

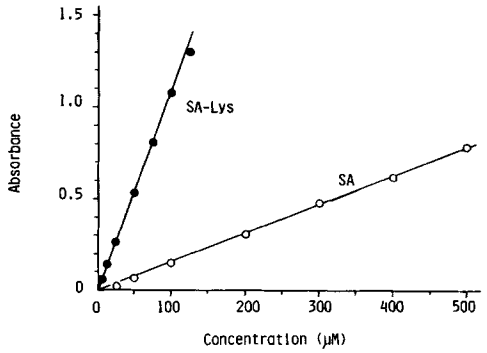

FIGURE 3. Absorbance of SA and SA-Lys as a function of their concentrations. SA-Lys adducts were prepared by incubating SA with Lys (100-fold excess) at pH 7.4 for 24 h. SA concentration was determined at 280 nm, while SA-Lys concentration was determined at 315 nm. SA-Lys concentration was expressed as the corresponding SA concentration.

Absorbance Spectra of SA-Albumin Adducts. In addition to amino acids, SA formed an adduct with purified human serum albumin that also displayed an absorbance maximum at 315 nm (data not shown). The absorbance at 315 nm increased linearly with time and reached a plateau at 24 h after incubation of albumin with SA at 37°C at pH 7.4.

Effects of pH on Stability of SA. To determine pH optima for the formation of SA adducts with amino acids and albumin, absorbance of the mixture was continuously recorded at 315 nm at various pH. All adducts were stable at pH between 7 and 8 for at least 1 mo at 37°C (data not shown). However, within 30 min, SA was completely released from the SA–amino acid adduct at a pH lower than 3. The SA-albumin adduct was more stable than SA–amino acid adducts at a low pH, with about 10% of the adduct remaining after 2-h incubation at pH 3.

SA adducts that had been reduced with $NaBH_4$ were more stable than the adduct without reduction. Incubation of the SA–amino acid adducts that had been reduced with $NaHB_4$ at pH 1.0 for 2 h resulted in a loss of 50% of the adducts. The reduced SA-albumin was even more stable than the reduced SA–amino acids, in that >60% of the adducts were present after incubation at pH 1.0 for 2 h.

Rate of Adduct Formation with SA. 20 amino acids were examined for their reaction with SA. All amino acids examined reacted with SA nonenzymatically, forming adducts as determined by the appearance of a new absorbance maximum at 315 nm. In addition to amino acids, SA formed a similar adduct with GSH. The rate of formation of the SA adduct with amino acids (40 mM) and GSH (40 mM) was determined at 20°C using an SA concentration of 1 mM. Representative results are shown in Fig. 4 for the SA adduct formation with Lys, glycine, methionine, phenylalanine, serine, alanine, glutamine, and GSH. The SA adduct formation was most rapid with Lys, followed in order by glycine, methionine, phenylalanine, GSH, serine, and alanine, and was slowest with glutamine. SA-adduct formation increased with time, and reached a maximum after 2 h with Lys (Fig. 4), but took 12 h with slow-reacting amino acids such as alanine.

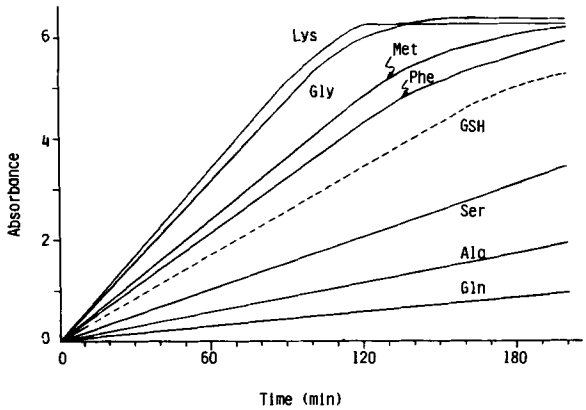

FIGURE 4. Rate of adduct formation of SA with amino acids as a function of incubation period. Absorbance at 315 nm was continuously recorded with time after 1 mM SA was added to each amino acid (40 mM) in 50 mM sodium phosphate buffer, pH 7.4, at 20°C.

Extraction of SA and SA–Amino Acid Adducts from Urine. To determine the rate of extraction of SA and SA-Lys from urine, they were extracted from 10 ml normal urine after supplementation with each compound (5 mM) using ethyl ether, followed by a mixture of chloroform/methanol (2:1 vol/vol). 67.5% of free SA was recovered at pH 1.0 and 29.4% at pH 7.4. 34.6% of SA-Lys was recovered at pH 7.4, however, little SA-Lys was recovered at pH 1.0, as expected. 64.5% of SA-Lys was recovered as free SA at pH 1.0. These results can be explained by the fact that SA-Lys is readily dissociated into free SA and Lys at pH 1. The results also suggest that urine samples extracted at pH 1.0 give a quantitative estimate of total SA, while urines extracted at pH 7.4 give a measure of the sum of free SA and SA–amino acid adducts.

HPLC Analysis of Free SA and SA–Amino Acid Adducts. An HPLC method was developed for the analysis of free SA and SA–amino acid adducts. As shown in Fig. 5*A*, three major peaks (A, B, and C) and two minor peaks were detected from a mixture of SA and Lys after incubation at pH 7.4 for 2 h, at 37°C, using absorbance at 315 nm. These peaks chromatographed entirely differently from free SA (Fig. 5*A* at 280 nm) and Lys (Fig. 5*A* at 210 nm), which were detected at each absorption maximum, i.e., 280 and 210 nm, respectively. The three major peaks (A, B, and C) had an identical absorption profile, with a peak at 315

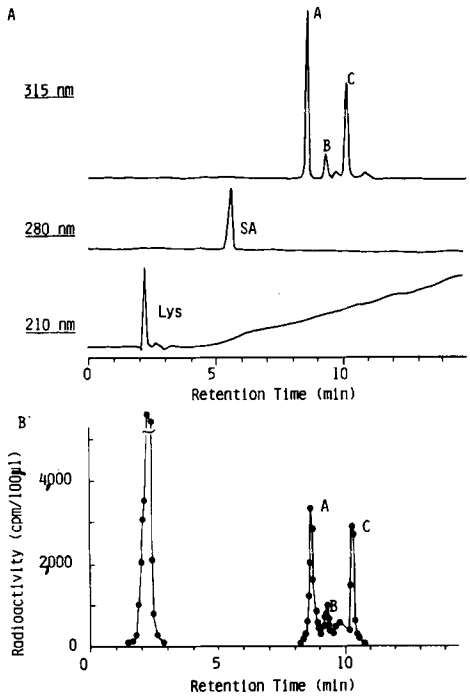

FIGURE 5. HPLC chromatographic profiles of SA, Lys, and SA-Lys. SA (50 mM) was incubated with 50 mM L-[^{14}C]Lys (sp act 324.7 mCi/mmole) at 37°C for 24 h. The incubated mixture was injected into HPLC as described in Materials and Methods. (*A*) 315 nm; SA-[^{14}C]Lys detected at 315 nm. 280 nm; SA detected at 280 nm. 210 nm: Lys detected at 210 nm. An increase in absorbance after the Lys peak is due to the absorption of methanol, which was used for gradient elution, at 210 nm. (*B*) Radioactivities of the above fractions (cf. Table I).

nm (not shown), suggesting that all three peaks represent SA-Lys adducts. Absorbance properties of the two minor peaks could not be examined, due to extremely low concentrations of these materials.

A chromatographic profile of a mixture of SA and [^{14}C]Lys is shown in Fig. 5*B*. The radioactive chromatographic profile is identical to the profile shown in the upper set, which had been examined at 210 and 315 nm. The molar ratio of SA/Lys in the SA Lys adducts was found to be ~1.0 (Table I). In addition, there was no significant difference in the elution profile between SA-Lys and SA-Lys that had been reduced with $NaBH_4$.

At 315 nm, SA-Lys adducts had significantly greater absorption than free SA, on a molar basis. There was also a linear relationship between the absorbance and the concentration of free SA or SA-Lys adducts. These properties allowed selective quantitation of free SA and SA-Lys adducts by HPLC.

HPLC Analysis of Urine Samples. Three patients with hereditary tyrosinemia, six other patients (two with Fanconi syndrome, two with nephrotic syndrome, and two with AIP), and six normal subjects were studied. SA–amino acids were detected in all 12 urine samples examined from the 3 patients with hereditary tyrosinemia. A typical chromatogram is shown in Fig. 6. An untreated urine specimen from a patient with tyrosinemia showed four major peaks (Fig. 6*A*), which corresponded to SA-glycine, SA-methionine, SA-tyrosine, and SA-phenylalanine (Fig. 6*C*). After digestion of the urinary sample with proteinase K, three more new peaks appeared on the chromatogram (Fig. 6*B*), three of which corresponded to SA-Lys (Fig. 6*C*). The fact that these peaks are derived from SA-Lys was also confirmed by collecting these peaks, and by reanalysis by TLC. In contrast to tyrosinemia, SA adducts were not detected in urines of normal subjects or patients with Fanconi syndrome, nephrotic syndrome, or AIP.

Chromatographic Identification of SA-Lys Derived from SA-Albumin in Tyrosinemia Urine. Albumin that had been incubated with excess SA at pH 7.4 for 24 h did not exhibit any HPLC peaks corresponding to SA–amino acid adducts, including that of SA-Lys (data not shown). In contrast, three peaks that are identical to SA-Lys (Fig. 6*C*) were detected in an SA-albumin preparation that had been digested with proteinase K (Fig. 7*A*). The fact that these three peaks were derived from SA-Lys was confirmed by rechromatography of these peaks by TLC (data not shown).

TABLE I
Molar Ratios of Lys to SA in SA-Lys Adduct

SA-Lys peak	Lys	SA	Lys:SA	Molar Lys:SA ratio
	cpm	*μmole*	*cpm/μmole*	
A	13,115	47.3	277	1.1
B	4,346	17.6	247	1.0
C	9,607	32.3	298	1.2

SA concentrations and radioactivities of [^{14}C]Lys (sp act 250 cpm/μmole) were determined in the three major fractions from SA-Lys adducts (*A*, *B*, and *C* in Fig. 5 *B*). After HPLC, SA was measured by converting the adduct to free SA and Lys by lowering pH to 1.0.

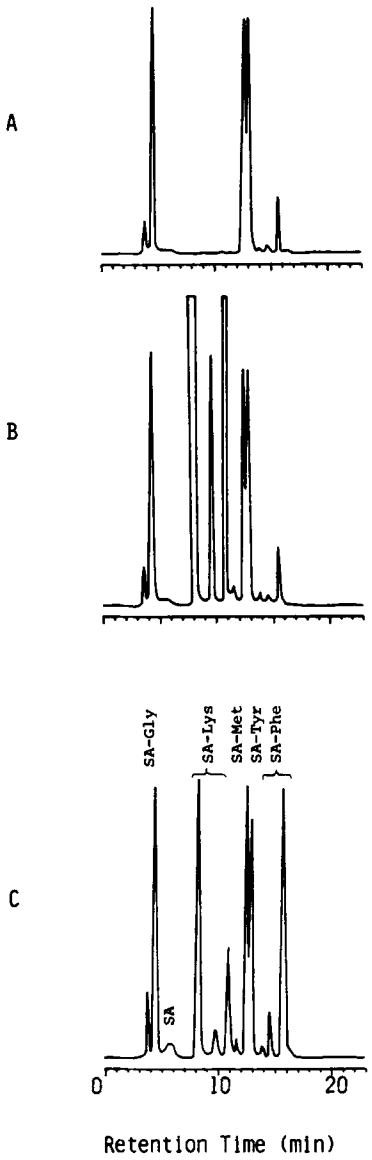

FIGURE 6. HPLC chromatographic profile of a urine sample from a patient with hereditary tyrosinemia. *A*, untreated urine; *B*, urine treated with proteinase K; *C*, mixture of SA–amino acid adducts. SA–amino acid adducts were prepared by incubation of SA with excess amino acids at pH 7.4 for 24 h at 37°C. Details for HPLC analysis and for incubation are described in Materials and Methods.

TLC offered a more extensive resolution of SA-Lys adducts than the HPLC method (Fig. 8). When the extract of SA-albumin that had been digested with proteinase K was rechromatographed by TLC, at least seven distinct bands derived from SA-Lys were detected. R_f for SA was found to be 0.60, and 0.58, 0.50, 0.39, 0.35, 0.25, 0.16, and 0.10 for SA-Lys (Fig. 8), while Lys remained at the origin in this system. The same bands as seen in the SA-Lys adducts were

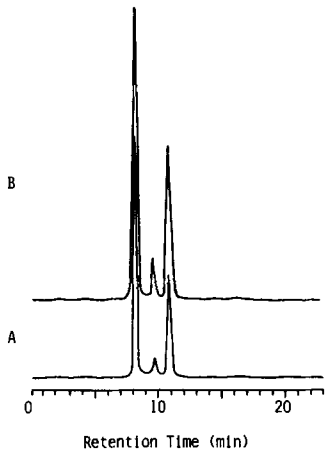

FIGURE 7. Demonstration of SA-Lys in SA-albumin after digestion with proteinase K. Human serum albumin (50 mg/ml) was incubated with or without SA (25 mM) at 37°C for 24 h. The mixture was then exhaustively dialyzed against 50 mM sodium phosphate buffer, pH 7.4. After reduction with $NaBH_4$, the mixture was incubated with proteinase K, at 37°C, for 2 h. *A*, SA-albumin after treatment with proteinase K; *B*, SA-albumin after treatment with proteinase K, mixed with SA-Lys. Nonincubated SA-albumin did not show any absorbance maxima under the condition of the assay (curve not shown).

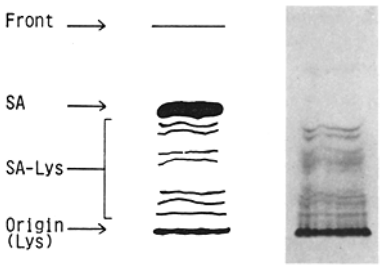

FIGURE 8. TLC separation of SA-Lys. SA-[^{14}C]Lys was chromatographed after reduction with $NaBH_4$. Compounds were visualized by iodide vapor (tracing on the left), and then autoradiographed (right).

seen in both SA-albumin and urine samples from patients with tryosinemia, both of which had been digested with proteinase K (data not shown). These results support the idea that SA binds to a Lys residue in albumin, and the three new peaks that appeared in the HPLC chromatogram after digestion of urine samples with proteinase K (Fig. 7*B*) are in fact SA-Lys derived from the SA-bound protein.

Effects of SA and SA-Lys on Activity of ALA Dehydratase. The effects of SA and SA-Lys on the activity of ALA dehydratase were examined using a homogenously purified enzyme from mouse liver. The enzyme reaction was carried out at pH 7.4 to assure the stability of SA-Lys. Under these conditions, both SA and SA-Lys inhibited the activity of ALA dehydratase, and the extent of enzyme inhibition by free SA (65% at 10^{-6} M) and SA-Lys (60% at 10^{-6} M) was almost identical.

Discussion

The results of this study show that SA reacts with amino acids and/or proteins such as albumin to form stable adducts at physiological pH and temperature. Lys is the most reactive amino acid with SA, among the 20 amino acids examined. These adducts all absorbed light at 315 nm and exhibited a significantly greater absorbance than free SA or amino acids at this wavelength. Lys residues in albumin also react with SA to form a stable adduct that can be identified by HPLC or by TLC after digestion of the protein with proteinase K. This finding suggests that chromatographic detection of the SA-Lys residue in the protein can be accomplished only after proteolytic cleavage of the protein.

Recently, Monnier and Cerami (11) showed that glucose can react with the amino groups of various proteins nonenzymatically to form stable adducts. The significance of this finding is critical, in that it provides a new approach to examine the nature of the crosslinking formed during aging of proteins and nucleic acids (12). SA also presumably forms an adduct with an amino acid or a protein via a Schiff base, since the adducts are stable at neutral pH, while they are quite unstable at low pH, e.g., pH 1. In addition, the adducts were made more stable after reduction with $NaBH_4$. Based on the absorbance of SA-Lys at 315 nm (molar absorbance = 1.09×10^4) (Fig. 3), the amount of SA bound per mole of albumin was calculated. In the human serum albumin solution (50 mg/ml = 750 μM) incubated with excess SA (25 mM) at 37°C for at least 24 h, the amount of SA bound was found to be 700 μM, indicating an approximately equimolar ratio of SA to albumin in the adduct. Since it is known that human serum albumin contains 58 Lys residues, the amount of Lys residues reacted with succinylacetone in the albumin was >2%. Reactivity of Lys in albumin is not uniform, in that nonenzymatic glycosylation of Lys of human serum albumin is known to occur only at Lys 525 (13). Whether Schiff base formation with Lys and SA also occurs at Lys 525 is not known, but SA probably only reacts with a highly reactive Lys in albumin.

SA in biological fluids occurring in patients with hereditary tyrosinemia has been determined either by gas chromatography (4, 5), or by inhibition of the activity of ALA dehydratase activity by adding urine or plasma from the patients to the enzyme preparation (7). However, the gas chromatographic method does not discriminate free SA from SA–amino acid adducts, and it was not clear whether the enzyme-inhibition assay discriminates free SA from SA-Lys adducts. Our data show that both free SA and SA-Lys equally inhibited the activity of ALA dehydratase, indicating that the enzyme inhibition assay does not discriminate the two forms of SA. In this study, we have developed new methods that can separately quantitate free SA and SA–amino acid adducts by spectrophotometry, TLC, or HPLC. Using these methods, we showed for the first time that SA-adducts with amino acids and/or proteins exist in biological fluids in patients with tyrosinemia. In fact, our data show that the majority of SA exists as SA–amino acids or SA-protein adducts in the urine from patients with this disease.

The spectrophotometric assay takes advantage of the fact that SA–amino acid adducts have a characteristic absorbance maximum at 315 nm, while free SA has a maximum at 280 nm, and other amino acids absorb between 260 and 280 nm. The molar absorbance of the SA–amino acid adduct at 315 nm was found to be

~10-fold greater than that of free SA. The minimal detection limit for free SA is ~13 μM, and it is 1.3 μM for SA-Lys by the spectrophotometric assay.

Our results show that all three patients with hereditary tyrosinemia examined excreted significant amounts of SA–amino acid adducts into their urine, while patients with other aminoacidurias, such as Fanconi syndrome and nephrotic syndrome, or patients with neurological abnormalities similar to those sometimes observed in tyrosinemia, such as AIP, did not show any detectable amounts of SA–amino acid adducts; neither did normal subjects. Since the majority of patients with tyrosinemia excrete large amounts of amino acids and/or proteins into their urine, it is likely that SA in their urine binds to amino acids or proteins nonenzymatically. Consistent with this view, we found that SA in the urine of patients with tyrosinemia exists, by and large, in the form of SA-protein adducts (average, 90%), while <10% exists as free SA. These results suggest that the spectrophotometric assay of SA adducts in the urine from patients might be very useful in screening for tyrosinemia.

Our HPLC method is able to separate free SA and each individual SA–amino acid in biological fluids in an unequivocal fashion. The resolution of free SA and individual SA–amino acid adducts is nearly complete, and is far more effective than the gas chromatographic separation of SA from other amino acids. Although the minimum detection limit for free SA and SA–amino acid at 280 nm is comparable, i.e., 0.75 pmole, SA–amino acid can be detected at 315 nm with an ~10-fold greater absorbance than at 280 nm. Separation of free SA and SA–amino acids can be further facilitated by using solvent extraction at different pH. If extracted at neutral pH, e.g., pH 7.4, SA–amino acid was preferentially recovered, while if extracted at pH 1, all SA (including SA in the adduct) is recovered quantitatively as free SA, since all SA–amino acids and SA-proteins are readily dissociated into free SA and amino acids or proteins at the low pH. Thus, the extraction at pH 1 and the determination at 280 nm can be used for the total estimation and SA in biological fluids, and the extraction at pH 7.4 with determination at 315 nm can be used for the quantitation of the sum of free SA and SA–amino acid adducts occurring at the physiological pH.

Using these procedures, the amount of free SA and SA–amino acid adducts in the urine of patients with tyrosinemia was estimated (Table II). When urine from patients with tyrosinemia was extracted at pH 7.4, without digestion with

TABLE II

SA Concentrations in Urine from Patients with Hereditary Tyrosinemia

Patient	SA in urine	
	With proteinase K	Without proteinase K
	μM	
J. O.	10.0	71.60
T. L.	0.80	42.45
M. O.	0.45	9.85

Urine samples with or without preincubation with proteinase K were analyzed by HPLC. Experimental details are described in Materials and Methods.

proteinase K, HPLC peaks corresponding SA-glycine, SA-methionine, SA-tyrosine, and SA-phenylalanine were detected. These peaks were not observed in urines from normal subjects, nor in patients with Fanconi syndrome, nephrotic syndrome, nor AIP. It should be noted that not only tyrosine, but also methionine and phenylalanine are known to be elevated in the sera of patients with tyrosinemia. If, however, the extraction was made at pH 7.4 after digestion with proteinase K in order to release the SA–amino acid adduct in the protein, three new HPLC peaks (Fig. 6*B*) corresponding to SA-Lys (Fig. 6*C*) were detected, in addition to the four SA–amino acid peaks that were present in the untreated urine from patients with tyrosinemia (Fig. 6*A*). This finding suggests that SA-Lys is derived from the Lys of proteins excreted into urine, presumably that of albumin. Moreover, significantly greater amounts of SA–amino acids (7- to 52-fold) were released from proteins in urine after digestion with proteinase K (Table II), suggesting that the majority of SA in the urine of patients with tyrosinemia is present in the form of protein-SA adducts.

TLC analysis also offered a rapid and simple separation of individual SA–amino acid adducts. As many as seven SA-Lys adducts could be identified after incubation of a mixture of SA and Lys at 37°C for 24 h (Fig. 8). Since [^{14}C]Lys used in this study is radiolabeled either at the α- or the ϵ-carbon position, eight different SA-Lys adducts could be hypothesized (Fig. 9).

As shown previously (9, 11), glucose and steroids form stable adducts with proteins, via a ketol moiety with the Lys residue in the protein. Since SA is a β-diketone that is highly reactive and, as shown in our study, reacts with amino

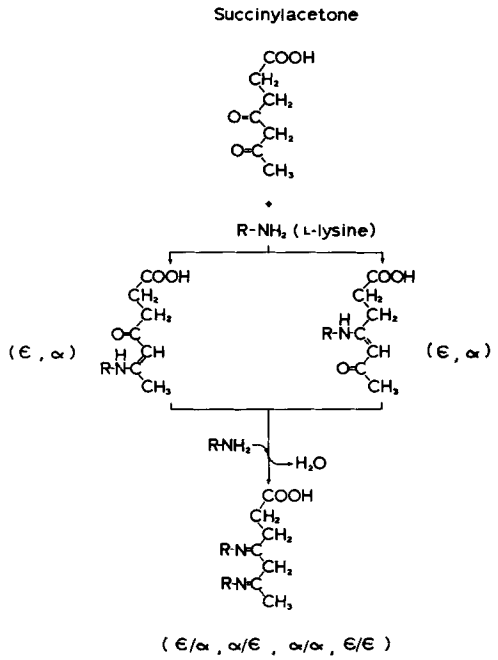

FIGURE 9. Possible structures of SA-Lys adducts. SA presumably binds with ϵ-amino group of L-Lys. Since [^{14}C]Lys used in this study is radiolabeled either at the α- or the ϵ-carbon position, eight different SA-Lys species could be formed.

acids and albumin in vitro, we expect SA to react with amino acids and proteins in plasma or urine in patients with tyrosinemia. For example, the reaction rate of SA with Lys is greater than that of glucose or steroids (cortisol and prednisolone) with amino acids (9, 11, 12). These findings support the idea that the majority of SA in tissues, plasma, and urine in patients with tyrosinemia exists in the form of SA-protein adducts.

It is known (1, 14) that treatment of patients with hereditary tyrosinemia with diets restricted in phenylalanine and tyrosine result in the reversal of the renal tubular dysfunction associated with the disorder. Renal tubular damage in these patients, as assessed by an increased excretion of β_2-microglobulin, appears to be associated with an increased excretion of SA and succinylacetoacetate, which ensues from the administration of phenylalanine and homogentisate (15). Since SA is highly reactive with proteins, it might also be responsible for the renal tubular damage in patients with tyrosinemia.

It has been reported (16) that the concentration of GSH in erythrocytes and in the liver in a patient with hereditary tyrosinemia was decreased. Our data indicate that SA reacts with GSH and forms a stable adduct (Fig. 4). Thus, excess SA produced in tyrosinemia may explain the apparent depletion of free GSH observed in patients with this disease.

The human hereditary diseases such as ataxia teleangiectasia, Fanconi's anemia, and Bloom's syndrome are characterized by increased cancer incidence and spontaneous chromosomal breakage (17). All these disorders are associated with abnormalities in oxygen metabolism (18), and the involvement of prooxidants in the carcinogenesis associated with these disorders has been postulated (19). The incidence of hepatoma is extremely high in patients with tyrosinemia (as many as 37% of the patients who survive early liver failure will develop hepatoma during childhood) (3). Since there is an indication of abnormal oxygen metabolism in tyrosinemia, as reflected in markedly reduced concentrations of hepatic GSH, it is possible that excess SA may, via the reduction of hepatic GSH, be responsible, in part, for the increased incidence of hepatoma in this disorder.

Summary

Succinylacetone (SA) (4,6-dioxoheptanoic acid) is an abnormal metabolite produced in patients with hereditary tyrosinemia as a consequence of an inherited deficiency of fumaryl acetoacetate hydrolase activity. Patients with this disease are associated with a number of abnormalities, including aminoaciduria, proteinuria, liver failure, commonly hepatoma, and decreased GSH concentration in the liver. In the course of our studies of tyrosinemia, we found that the urine of patients with this disorder contains material(s) that absorbs light at 315 nm. We investigated the nature of the 315 nm material in detail. SA was found to react with amino acids and protein nonenzymatically, to form stable adducts at physiological temperature and pH. All SA adducts with amino acids and/or proteins exhibited an absorption peak at 315 nm. Although all amino acids reacted with SA, the most reactive amino acid was lysine (Lys), followed, in order, by glycine, methionine, phenylalanine, serine, alanine, and glutamine. SA-adducts were unstable at pH below 6, while they were made considerably more stable after reduction with $NaBH_4$, suggesting that SA forms an adduct via Schiff base

formation. High-performance liquid chromatography (HPLC) analysis of urines from patients with tyrosinemia revealed the existence of SA-glycine, SA-methionine, SA-tyrosine, and SA-phenylalanine. After digestion of urines with proteinase K, three more HPLC peaks appeared, which all corresponded to SA-Lys adducts. TLC analysis of SA-Lys showed that SA-Lys could form as many as seven different adducts. No SA-adduct peaks were observed in HPLC in urines from normal subjects, patients with other forms of aminoaciduria, or patients with the nephrotic syndrome. In addition to amino acids and proteins, SA reacted with reduced glutathione (GSH) and formed a stable adduct. These findings suggest that SA adduct formation with amino acids, GSH, and proteins is a significant process occurring in tyrosinemia, and may account for certain of the pathologic findings in this hereditary disorder.

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