Mammalian Utilization of Cutin, the Cuticular Polyester of Plants

Radioactive apple fruit cutin ingested by rats was hydrolyzed in the intestine, and the products were absorbed and metabolized. Evidence is presented which suggests that a pancreatic enzyme was involved in the hydrolysis of this polyester, composed of hydroxy and epoxy fatty acids. The results suggested that biopolyesters, which constitute a major component of wastes from fruit processing, can provide caloric value to animal diets.

Cutin, the structural component of plant cuticle, is an insoluble biopolyester and constitutes a major component of the waste material generated by the fruit processing industry. Analyses of the depolymerization products of cutin from a variety of plants by GLC–MS revealed that the major components of the polymer are C_{16} and C_{18} fatty acids, ω-hydroxy fatty acids, dihydroxy C_{16} acids, 18-hydroxy-9,10-epoxy C_{18} acids, and 9,10,18-trihydroxy C_{18} acids (Kolattukudy, 1977). A related polymer, suberin, is the major component of the skin of potato tubers and other root crops, and it is a major waste material generated in the processing of such crops. These waste materials, which also contain portions of the internal tissues, are generally used as cattle feed and they are of major importance as feedstuffs for finishing cattle in the western states (Wilson et al., 1970; Heinemann and Dyer, 1972). Even though the commercial by-products are known to have some nutritional and caloric value, nothing is known about the fate of the polymeric materials of the plant skin contained in these agricultural waste products. There are indications that this biopolyester (cutin) is fairly resistant to biodegradation. For example, analysis of sewage sludge showed that it contained substantial amounts of cutin (Kolattukudy and Purdy, 1973). Cutin has been recovered even from ancient sediments (Eglinton et al., 1968). On the other hand, fungal (Heinen and Devries, 1966; Shishyama et al., 1970; Purdy and Kolattukudy, 1973) and bacterial (Hankin and Kolattukudy, 1971) degradation of cutin has been demonstrated and cutin hydrolyzing enzymes have been isolated and characterized from fungi (Purdy and Kolattukudy, 1975a,b; Soliday and Kolattukudy, 1976). Even though cutin is a component of the diet of not only livestock but also of humans, it is not known whether this polyester is utilized by animals. Previous in vitro measurement of cutin degradation by bovine rumen was reported to have given negative results (Chatterton and Powell, 1974).

In this paper we show that labeled apple cutin ingested by rats is hydrolyzed in the intestine, and the products are absorbed and metabolized.

EXPERIMENTAL SECTION

Materials. Male Sprague-Dawley rats were obtained from the animal resource center of this university. Cellulase (*Aspergillus niger*), pectinase (fungal) lithium aluminum hydride, chloramphenicol, and biosal were purchased from Sigma Chemical Co. SilicAR CC4 (silicic acid) was from Mallinckrodt Inc. Aquasol was from Fisher, and Omnifluor and [1-^{14}C]acetate were purchased from New England Nuclear Corp.

Preparation of ^{14}C-Labeled Cutin. Skin slices (11 mm diameter) were prepared from young McIntosh apple fruits (3–4 cm diameter) and these disks were incubated with [1-^{14}C]acetate for 6 h at 30 °C (125 μCi, 2.1 μmol/25 disks) as described previously (Purdy and Kolattukudy, 1973). After thoroughly washing the disks with water, 400 disk batches were placed in 1500 mL of 50 mM acetate buffer, pH 4.0, containing 7.5 g of cellulase and 1.5 g of pectinase. After gentle swirling at 30 °C overnight, the separated disks of cuticle were collected, and the adhering fruit tissue was carefully scraped away from each cuticular disk. These disks were soaked in an excess of a 2:1 mixture of $CHCl_3$ and CH_3OH, thoroughly washed with the same solvent mixture, and then Soxhlet extracted with $CHCl_3$ overnight to remove the cuticular wax. The enzyme treatment and $CHCl_3$ extraction were repeated and the labeled cutin disks were stored dry at −20 °C. The amount of ^{14}C incorporated into the cutin was determined in the following manner. A few randomly selected disks were homogenized (Ten-Broeck) in tetrahydrofuran, and the suspension was refluxed with an excess of $LiAlH_4$ overnight. The excess $LiAlH_4$ was decomposed with water, the mixture was acidified with HCl, the lipid products were recovered by extraction with $CHCl_3$, and an aliquot was assayed for ^{14}C. The aqueous phase which was also assayed for ^{14}C contained <5% of the label contained in the organic solvent.

Feeding Experiment. A mature, male Sprague-Dawley rat (325 g) was starved overnight and then fed 6.5 g of a food mixture prepared as follows. Labeled apple cutin (66.1 mg, 5.2×10^6 cpm) was homogenized (Ten-Broeck) in acetone and centrifuged at 12 000g for 10 min. The supernatant was decanted and the pellet was washed twice more with acetone and then three times with deionized water in a similar manner. Ground rat chow (2.59 g, 60 mesh) was added and the mixture was formed into a cohesive cake with the addition of a small amount of a 4% agar solution. After feeding (1 h), the rat was placed in a dessicator fitted with an inlet for compressed air and an outlet which was attached to a gas dispersion tube submerged in 10% KOH (w/v) in order to absorb the expired $^{14}CO_2$. Aliquots of the KOH solution were taken hourly and assayed for ^{14}C. When the release of $^{14}CO_2$ nearly ceased, the rat was killed by passing carbon monoxide into the inlet line. Immediately after death, the rat was dissected, and samples of various organs, tissues, and excretions were collected and aliquots were assayed for ^{14}C.

The liver, feces, intestinal contents, and intestinal wall (well washed with 0.9% (w/v) saline) were homogenized (Ten-Broeck) in $CHCl_3$–CH_3OH (2:1 v/v) and the lipids were extracted by the method of Bligh and Dyer (1973). The urine was acidified with HCl, and the lipids were recovered by extraction with diethyl ether. Small aliquots of the extracted lipids were assayed for ^{14}C and the remaining lipid samples were refluxed overnight with $LiAlH_4$ in tetrahydrofuran and the hydrogenolysis products were recovered by $CHCl_3$ extraction as indicated above. The $CHCl_3$-soluble products thus obtained from urine, feces, and intestinal contents were subjected to thin-layer chromatography on 0.5 mm silica gel G plates with ethyl ether–hexane–methanol (8:2:1 v/v) as the developing solvent. Hydrogenolysis products of apple cutin was used as an internal standard, and the thin-layer chromatographic fractions were visualized under UV light after spraying the plates with a 0.1% (w/v) ethanolic solution

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of dichlorofluorescein. The silica gel from the various regions were scraped into counting vials and assayed for ^{14}C by liquid scintillation spectrometry. The hydrogenolysis products of the lipids from the liver and intestinal wall samples were first subjected to column chromatography with silicic acid. Each sample was applied to a column (2.2 × 50 cm) of SilicAR CC-4 packed in hexane. The nonpolar lipids were eluted with 1 L of $CHCl_3$. The more polar hydroxyalkanes derived from the hydroxy acids of cutin were eluted with 1 L of methanol. The latter fraction was subjected to thin-layer chromatography as described above.

Preparation of Pancreatic Acetone Powder. Pancreases were removed from freshly killed rats, trimmed of fat, and homogenized (Ten-Broeck) in acetone (−20 °C). The suspension was immediately filtered with a Buchner funnel and the acetone powder was dried overnight in vacuo. Portions of the acetone powder were stirred with 0.1 M Tris–HCl buffer, (≃5 mg/mL) pH 7.0 at 0–4 °C for 1 h, and the mixture was centrifuged at 27 000*g* for 15 min; the supernatant was used as the enzyme source.

Measurement of Enzymatic Cutin Hydrolysis. The labeled cutin disks were homogenized (Ten-Broeck) in acetone and mixed with similarly homogenized unlabeled apple cutin so that the desired specific activity was obtained (usually 50 000 cpm/3 mg) and the suspension was centrifuged at 20 000*g* for 5 min. The solid material recovered was washed twice more with acetone, three times with deionized water, and finally with the assay buffer, usually 0.1 M Tris–HCl, pH 7.5, each time the solid was suspended in the solvent for 5 min and centrifuged.

Appropriate aliquots of enzyme solution were added to 0.1 M Tris–HCl, pH 7.5, containing 3 mg of labeled cutin (50 000 cpm) in a total volume of 0.5 mL, and the reaction mixture was incubated in a gyrating water bath at 30 °C for 4 h. The reaction was terminated by force filtration of the reaction mixture through a glass wool plug in a pasteur pipet, followed by a 0.5 mL of deionized water and two 0.5-mL portions of acetone (to wash through any noncovalently bound monomers). Aliquots of the filtrate were assayed for ^{14}C, and the amount of radioactivity released was taken as a measure of enzymatic hydrolysis of cutin. Controls were run with identical reaction mixtures without enzyme and these values were subtracted from the experimental values.

Determination of Radioactivity. Liquid scintillation spectrometry was used to assay for ^{14}C in all samples. Aquasol (when the aqueous aliquot to be assayed was >0.2 mL) or 0.4% (w/v) Omnifluor dissolved in toluene containing 30% ethanol was used as the counting fluid. Internal standards ([^{14}C]toluene) were used to determine efficiency, which was usually about 70%. Thin-layer chromatographic fractions were scraped into counting vials, mixed with the Omnifluor containing fluid, and assayed for ^{14}C. All counting was done with a standard deviation <3%.

RESULTS AND DISCUSSION

Feeding Experiments. Within 1 h after ingesting the labeled cutin, $^{14}CO_2$ could be detected in the exhaled air; $^{14}CO_2$ output increased up to about 6 h and subsequently leveled off. Therefore the tissues and excretions of the animal were examined about 7 h after ingesting labeled cutin (Table I). The fact that every organ and tissue examined contained ^{14}C and that liver contained much larger amounts of ^{14}C than any other tissue (Table I) strongly suggested that labeled monomers from the polymer were released in the intestinal tract of the animal, absorbed, and catabolized. That extensive metabolism of the monomers occurred is indicated by the observation that a significant proportion (>7%) of the label from the ingested polymer was found in the expired CO_2 even though intestinal contents and feces contained fairly large amounts of ^{14}C. The most highly labeled samples were further examined to determine the amount of ^{14}C present in the lipids and water-soluble components. Substantial proportions of the label in the liver, intestinal wall, urine, and blood samples were found in the water-soluble metabolites, suggesting that extensive catabolism of the absorbed monomers occurred (these water-soluble products were not further examined). A high proportion of the label in the intestinal contents and feces was found in chloroform-soluble lipids and the remaining portion was in insoluble materials obviously representing unhydrolyzed cutin. These observations suggest that substantial amounts of the labeled polymer ingested by the animal were hydrolyzed in the intestinal tract but a significant portion of the hydrolysis products was not absorbed.

To determine whether the radioactivity found in the lipids extracted from the various parts of the animal represented unaltered cutin monomers or lipids resynthesized from the degradation products of cutin acids, the extracted lipid fractions were reduced with $LiAlH_4$ (hydrogenolysis) and the products were analyzed by thin-layer chromatography (Walton and Kolattukudy, 1972). This technique gave five major classes of reduced monomers from the labeled apple cutin: (1) the fatty alcohols (R_f 0.76) derived primarily from the C_{16} and C_{18} fatty acids; (2) the diols (R_f 0.49), from 16-hydroxypalmitic acid, 18-hydroxystearic acid, and 18-hydroxyoleic acid; (3) the C_{18} triols (R_f 0.39), from 18-hydroxy-9,10-epoxystearic acid and its unsaturated analogues; (4) the C_{16} triols (R_f 0.27), from 9,16- and 10,16-dihydroxypalmitic acid; and (5) the tetraols (R_f 0.22), from 9,10,18-trihydroxystearic acid and its unsaturated analogues. The lipids extracted from feces, intestinal contents, and intestinal wall upon hydrogenolysis gave labeled monomers with ^{14}C distribution characteristic of apple cutin (Kolattukudy et al., 1973), suggesting that these fractions did contain intact cutin acids which had not undergone degradation (Table II). In further support of this conclusion was the observation that in these three cases the bulk (~90%) of the label contained in the original lipid extract was recovered in the $CHCl_3$ extract of the hydrogenolysate. In the case of urine, two-thirds of the total radioactivity was in water-soluble products and upon hydrogenolysis of the $CHCl_3$ extract of urine, 60% of the label contained in the original $CHCl_3$ extract was found in water-soluble products. Thus, only about 13% of the label in urine was recovered in aliphatic chains, and although a significant portion of this label was found in the monomer fractions expected from cutin, this small amount could represent contamination from fecal material. Thus, the label in urine represents mainly water-soluble excretion products generated by metabolism of cutin monomers. Analysis of the hydrogenolysis products of the lipids from liver and blood samples showed that only 60 and 36% of the label, respectively, was present in aliphatic chains, the remaining portion was found in the water-soluble products. Thin-layer chromatographic analyses of the $CHCl_3$ soluble hydrogenolysis products showed that about 90 and 70% of the label contained in these products derived from liver and blood, respectively, was recovered in the fatty alcohol fraction and only the remaining small portion was in the more polar products. These results suggest that the label found in the lipids of blood and liver represented mainly products resynthesized from the catabolic products of labeled cutin monomers. The dis-

Table I. Distribution of ^{14}C in a Rat Which Ingested Apple Fruit Cutin Derived from [1-^{14}C]Acetate[a]

tissue or waste product	radioactivity, 10^3 cpm: total fraction	lipid extract	aqueous phase
carbon dioxide	363.0		
intestinal contents	1036.0	546.0	75.0
feces	576.0	327.0	29.0
urine	413.0	139.0	274.0
blood[b]	22.5	3.0	19.5
stomach contents	319.0		
intestinal wall	506.0	135.0	75.0
stomach	45.6		
liver	619.0	80.4	324.0
kidneys	78.6	14.5	
heart	6.7		
lungs	14.6		
brain	14.6		
spleen	12.5		
testes	17.9		
haunch muscle[c]	12.4		
back muscle[c]	5.7		
abdominal muscle[c]	2.6		
subcutaneous fat from neck[c]	2.3		
abdominal fat pad[c]	2.0		

[a] A 325-g male rat was fed 66.1 mg of labeled cutin (5.2×10^6 cpm) mixed with 2.59 g of rat food. The $^{14}CO_2$ output nearly ceased in 7 h and the rat was sacrificed. The insoluble materials from the fractions were not further examined. [b] Total radioactivity in 4.5 mL of blood recovered from heart immediately after killing. [c] Radioactivity per gram of tissue.

tribution of ^{14}C in the various parts of the animal and the nature of the labeled components found in the various fractions clearly suggested that the labeled apple cutin, ingested by the rat, was hydrolyzed in the intestinal tract, absorbed, and transported to the liver, which appears to be the major site of catabolism of the hydroxy acids.

In order to determine whether the hydrolysis of the ingested polymer was catalyzed by some enzyme excreted by the rat or by microbial flora, three types of experiments were performed, and the results strongly suggested that microbial flora was not the major source of the cutin hydrolyzing activity in the intestinal tract of the rat. (a) Feeding experiments similar to that described above were performed with a rat which had been previously given chloramphenicol (36 mg/100 mL) with Biosal (4 drops/100 mL) in its drinking water for 4 days to lower the bacterial flora of the intestine to <1% of that originally present. Such treatment, however, had little effect on the amount of hydrolysis, and the subsequent absorption and metabolism of the ingested cutin. (b) When the intestinal contents of a rat was suspended in isotonic buffer (50 mM Tris–HCl pH 8, 0.3 sucrose) and centrifuged at 20 000*g* to remove bacteria, all of the cutin hydrolyzing activity was recovered in the supernatant, suggesting that a soluble enzyme present in the intestinal content was responsible for cutin hydrolysis. (c) The small intestine of a rat was removed and cut into six 20-cm sections, the first beginning just below the stomach and the last ending at the colon. The contents of each section were homogenized and assayed for cutin hydrolyzing activity as described in the methods section using 400 000 cpm for each assay. If bacterial flora were responsible for cutin hydrolysis, a higher amount of hydrolytic activity would be expected in the lower end of the intestine where microbial flora are more prominent. The results, however, showed nearly uniform distribution of hydrolytic activity (releasing 14 to 19.6% of the ^{14}C from the polymer) along the first five sections and the last section near the colon had less hydrolytic activity (released 11% of the ^{14}C from the polymer), suggesting that it is unlikely a bacterial protein was responsible for cutin hydrolysis.

Table II. Thin-Layer Chromatographic Analyses of the Hydrogenolysis ($LiAlH_4$) Products of Lipids from the Various Regions of a Rat Which Ingested Labeled Apple Fruit Cutin

sample	radioactivity, %: fatty alcohol	diol	C_{18} triol	C_{16} triol	C_{18} tetraol
apple cutin in the diet	1.2	29.4	13.9	44.1	11.4
intestinal content	10.7	24.8	13.9	38.8	11.8
feces	2.5	17.8	17.7	40.9	21.0
intestinal wall	5.6	31.9	13.7	27.4	21.4
urine[a]	9.5	17.7	30.0	22.8	20.0
blood[b]	68.8	12.7		14.0	4.5
liver[b]	91.2	4.2		3.2	1.3

[a] Might have contained some fecal material; the C_{16} triol and C_{18} triol were not completely resolved. [b] The C_{16} triol and C_{18} triol fractions were taken together for counting as they were not fully resolved.

Pancreas as the Source of Cutin Hydrolyzing Activity. Analyses of the feces and intestinal contents of the rat, which ingested labeled cutin, revealed that the bulk of the radioactivity was present in the polymeric material and hydrolysis products but not much in products (*n*-fatty acids and water-soluble products) of further catabolism of cutin monomers. These results are in agreement with the conclusion that within the intestine only hydrolysis of the polymer (but not degradation of the monomers) occurred. Preliminary characterization of cutin hydrolysis catalyzed by the 20 000*g* supernatant prepared from the intestinal content showed that all classes of monomers were released by the enzyme present in the intestine. The amount of ^{14}C released into soluble lipids was proportional to the amount of protein added and maximal hydrolysis was obtained at around pH 8. These results together with the observation that the hydrolytic activity was found in the small intestine but not in the stomach led us to suspect that a pancreatic enzyme was responsible for cutin hydrolysis in the rat. In fact, a buffer extract of acetone powder prepared from rat pancreas catalyzed hydrolysis of labeled apple cutin (50 mg of acetone powder was suspended in 3 mL of 50 mM Tris–HCl, pH 8, at 4 °C for 1 h and the 25 000*g* supernatant released about one-third of the ^{14}C contained in 3.6 mg of apple cutin in 4 h at 30 °C). Thin-layer chromatographic analysis of the hydrogenolysate of the soluble material, released by the pancreatic preparation, showed that the radioactivity distribution among the various monomer classes was similar to that in the intact cutin shown in Table II. The pancreatic enzyme preparation showed a pH dependence profile identical with that observed with the intestinal enzyme with pH optimum at 8.0. These results strongly suggested that the pancreas was the source of the enzyme responsible for cutin hydrolysis in the rat. It is possible that one of the known hydrolases excreted by the pancreas such as a lipase is responsible for the hydrolysis of cutin.

In view of the report that microscopic studies (Chatterton and Powell, 1974) showed that cutin was not digested by the bovine rumen, the present experiments were designed to use a sensitive technique to test whether this polyester can be digested by animals. The results presented in this paper show that a substantial portion of cutin ingested by the rat was hydrolyzed, absorbed, and me-

tabolized. Until much more extensive feeding studies are performed, it is not possible to determine the cutin content of the diet, which will give optimum utilization of this biopolymer. The present finding that rats do possess the ability to digest cutin and utilize the monomers released, strongly suggests that the waste products of fruit processing, which is rich in the fruit skin (cutin) can provide significant caloric value to animals. Similarly, a related polymer, suberin, in the potato peel can also probably provide caloric value to animals and thus these waste materials can be useful components of animal feeds. If long-term feeding of such materials would result in the establishment of adaptable microorganisms in the digestive tract, higher utilization of such waste materials might result particularly in the ruminants.

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