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The Identification and the Excretion Pattern of Isovaleryl Glucuronide in the Urine of Patients with Isovaleric Acidemia

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Summary

We identified isovaleryl glucuronide in the urine of patients with isovaleric acidemia by using gas chromatography-mass spectrometry (GC-MS) and by identifying the products of enzymatic hydrolysis. Conjugation of isovaleryl-CoA with glycine, by the action of glycine-*N*-acylase, is the main detoxification mechanism in isovaleric acidemia. The identification of isovaleryl glucuronide demonstrates a hitherto unknown, additional detoxification mechanism in patients with isovaleric acidemia. Quantitative analysis of 72 urine specimens from four patients with isovaleric acidemia shows that isovaleryl glucuronide is more likely to be excreted when the amount of urinary 3-hydroxyisovaleric acid excretion is high. This suggests that detoxification via glucuronide conjugation plays an important role when the glycine conjugation system is saturated.

Abbreviations

a.m.u., atomic mass unit
BSA, N,O-bis(trimethylsilyl)acetamide
CI, chemical ionization
EI, electron impact
GC, gas chromatography
MS, mass spectrometry
TMS, trimethylsilyl

Isovaleric acidemia is a recessively inherited disorder of leucine metabolism, resulting from a deficiency of isovaleryl-CoA dehydrogenase (7, 8). Clinical manifestations include acute attacks of vomiting, acidosis, ataxia, lethargy, and coma (1, 11). The enzyme block leads to the accumulation of isovaleryl-CoA in tissues. During periods of remission, unoxidized isovaleryl-CoA is handled by conjugation with glycine, catalyzed by the action of glycine-*N*-acylase. Isovalerylglycine thus formed is disposed by urinary excretion (9). When this system is saturated, excess isovaleryl-CoA is hydrolyzed and released as free isovaleric acid causing acute toxic effects. Free isovaleric acid is then omega-1 oxidized or omega oxidized to 3-hydroxyisovaleric (10) or 4-hydroxyisovaleric (13) and methylsuccinic (13) acids, respectively. Small fractions of isovaleryl-CoA are also metabolized to other unusual organic acids including 3-hydroxyisoheptanoic (4) and isovalerylglutamic acid (5).

During the last several years we have identified four patients with isovaleric acidemia by GC and GC-MS. We subsequently analyzed many follow-up samples from these patients. While analyzing these samples, we noticed a well-defined peak which eluted at very high temperatures (methylene unit 22.27 on 10% OV-1) in a number of these samples, in amounts ranging up to 0.50 mg/mg creatinine. In this report, we describe the identification of this compound as isovaleryl glucuronide using GC and GC-MS as well as enzymatic methods.

MATERIALS AND METHODS

Chemical materials. "Tri Sil-BSA Formula P" was purchased from Pierce Chemical (Rockford, IL). High purity *E. coli* β-

glucuronidase type VII was purchased from Sigma Chemical Co. (St. Louis, MO). Coiled glass columns (2 mm x 6 ft) were obtained from Supelco, Inc. (Bellefonte, PA). All other chemicals were reagent grade and obtained from standard commercial sources.

Instrumentation. Gas chromatography was performed using a Hewlett-Packard 5840 gas chromatograph equipped with dual flame ionization detectors and a data system. TMS samples for organic acids were analyzed using a glass column packed with 3% OV-1 on 100/200 mesh Gas Chrom Q from Applied Science Laboratories (State College, PA). Injection port and detector were kept at 225° and 300°C, respectively. The column temperature was programmed from 70–260°C at 6°C/min. The nitrogen carrier gas flow rate was 25 ml/min. Short chain fatty acid samples were analyzed using a glass column packed with "Carbopack C/0.3% Carbowax 20M/0.1% Phosphoric acid" from Supelco, Inc. Injection port and detector were kept at 150° and 200°C, respectively. The column temperature was held isothermally at 120°C. The nitrogen carrier gas flow rate was 25 ml/min.

A Finnigan 4510 GC-MS-COM was used for mass spectral studies. Capillary GC was used as the inlet using a 0.25 mm x 30 m column coated with DB-5 (SE-54) from J and W Scientific, Inc. (Rancho Cordova, CA). Helium was used as the carrier gas. The split injection mode was used with a flow rate of approximately 1 ml/min. The capillary column temperature was programmed from 70°–270°C at 5°C/min, after an initial hold of 5 min. The injection port, separator, and transfer line were kept at 225°, 250°, and 270°C, respectively. The ionizing voltage was 70 eV for electron impact analysis and methane was used as reagent gas for chemical ionization analysis.

Sample preparation. Urinary organic acids were analyzed as TMS derivatives as described previously (12). Spot urine samples from patients with isovaleric acidemia were stored at −20°C until analysis.

For short chain fatty acid analysis, an aliquot of urine was adjusted to pH 3 by the addition of 0.5 ml of saturated K phosphate buffer, pH 2.90. Five drops of isoamyl alcohol were added as an anti-foaming agent. The sample was transferred to a micro-steam distillation apparatus. Ten milliliters of distillate was collected per 1 ml of sample. The pH of the distillate was adjusted to >10 by the addition of 0.5 ml of 0.1 N NaOH. The sample was evaporated to dryness with a nitrogen stream in a water bath at 100°C. One-tenth milliliter of 10% formic acid was added to redissolve the residue and adjust the pH to <3. One microliter of the sample was injected into the GC.

Hydrolysis of isovaleryl glucuronide with β-glucuronidase. Preweighed vials containing 1000 U of high purity β-glucuronidase type VII from *E. coli* were used. When reconstituted with 10 ml of water the solution contained 4 mM phosphate buffer pH 6.8. One milliliter of the enzyme solution was added to 1 ml of urine. As a control, 1 ml of 4 mM phosphate buffer, pH 6.8, was added to another equal aliquot of the same urine. The samples were incubated in a water bath at 37°C for 1 h. After incubation the samples were divided into two equal aliquots. One aliquot was extracted with ethyl acetate for organic acid analysis as TMS derivatives and the other aliquot was steam distilled for short chain fatty acid analysis.

RESULTS AND DISCUSSION

Gas chromatographic detection and mass spectral identification. A typical GC chromatogram of the urine from a patient with isovaleric acidemia, which contained an unidentified, late eluting peak, is shown in Figure 1. This chromatogram was from analysis performed by our routine screening method using a packed 10% OV-1 column. This sample was subsequently analyzed by GC-MS-COM using a DB-5 capillary column, and the EI and CI spectra obtained are shown in Figures 2 and 3, respectively. The majority of the ions in the EI spectra, with the exception of those at m/e 551, 85, and 57, are characteristic of

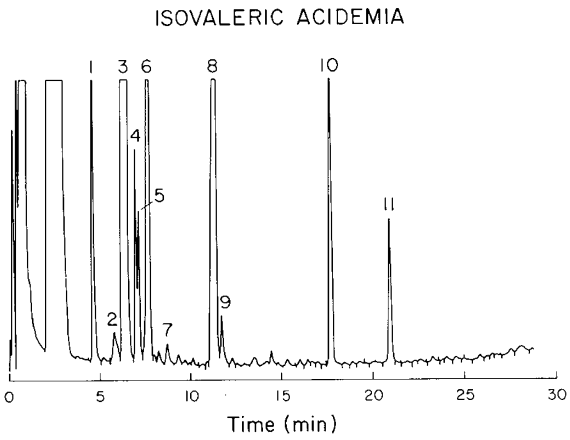

Fig. 1. Gas chromatogram (GC) of an isovaleric acidemia urine sample. A 10% OV-1 column was used for analysis. The numbered GC peaks were identified by GC-mass spectrometry as: *1*) lactic acid; *2*) 2-hydroxybutyric acid; *3*) 3-hydroxybutyric acid; *4*) acetoacetic acid (peak 1); *5*) 3-hydroxyisovaleric acid; *6*) acetoacetic acid (peak 2), *7*) succinic acid; *8*) isovalerylglycine; *9*) adipic acid; *10*) pentadecanoic acid (internal standard); and *11*) isovaleryl glucuronide.

glucuronide conjugate TMS derivatives (6). These ions characteristic for glucuronides are the ions at m/e 464, 449, 375, 359, 333, 319, 305, 257, 217, 204, 169, 147, 129, 117, 103, and 95 (6). Glucuronide conjugate TMS derivatives usually do not produce a molecular ion in EI and frequently fail to do so in CI as well. We therefore suspected that the ion at m/e 551 corresponded to M-15. The CI spectra supports this assumption because the ion at m/e 551 is of higher intensity and is not shifted 1 a.m.u. higher as would be if it were the molecular ion. For comparison, we ran another sample which was known to contain di(propyl)acetate (valproate) glucuronide TMS derivative under the same CI conditions and this compound also produced an M-15 ion and no molecular ion. Ions at m/e 85 and 57 are prominent in the EI spectra of the unknown. Given the biochemical nature of the disease and the general fragmentation pattern of glucuronide conjugates we postulated that the ion at m/e 85 corresponded to an isovaleryl group and the ion at m/e 57 corresponded to the isobutyl portion of the isovaleryl group as illustrated in Figure 4. The molecular weight of isovaleryl glucuronide tetra-TMS is 566 and hence the ion at m/e 551 would correspond to M-15 as suspected; thus, we identified the unknown as isovaleryl glucuronide based on the mass spectral analysis.

Studies with enzymatic hydrolysis. In order to confirm the identification of isovaleryl glucuronide, we analyzed an isovaleric acidemia urine sample which was pretreated with highly purified β-glucuronidase from *E. coli* for short chain fatty acids and organic acids as TMS derivatives. This sample contained 150 μg/ml (0.54 μmol/ml) of isovaleryl glucuronide as determined by GC. As a control we analyzed another aliquot of the same urine that had been treated with the same phosphate buffer alone. The peak identified as isovaleryl glucuronide was unchanged in the sample treated with buffer alone but it was absent in the sample treated with β-glucuronidase. Otherwise, the organic acid profile including isovalerylglycine was essentially identical quantitatively, indicating high specificity of glucuronidase action. The chromatograms obtained for short chain fatty acid analysis are shown in Figure 5. The amount of isovaleric acid greatly increased in the sample treated with β-glucuronidase. The identity of isovaleric acid was confirmed by coinjection of authentic isovaleric acid. The additional isovaleric acid liberated by glucuronidase treatment was calculated to be 67 μg/ml (0.66 μmol/ml) which is 22% higher than the estimated amount of isovaleryl glucuronide. This small discrepancy was determined to be due to the fact that isovaleryl glucuronide was not quantitatively recovered by ethyl acetate extraction which was utilized

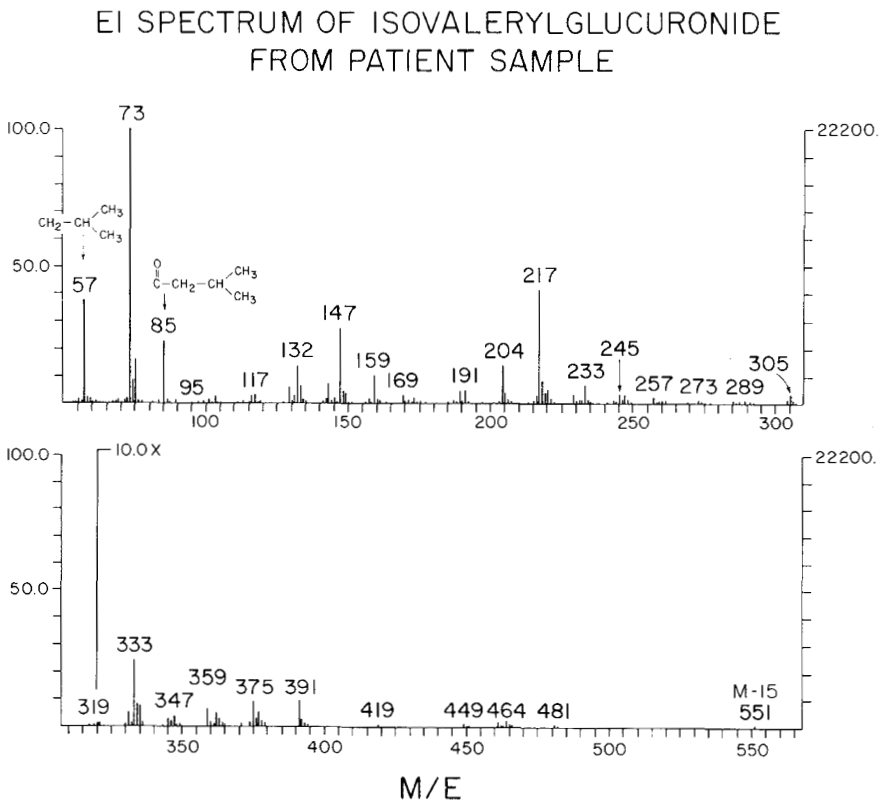

Fig. 2. Electron impact mass spectrum of gas chromatography peak #11 from Figure 1.

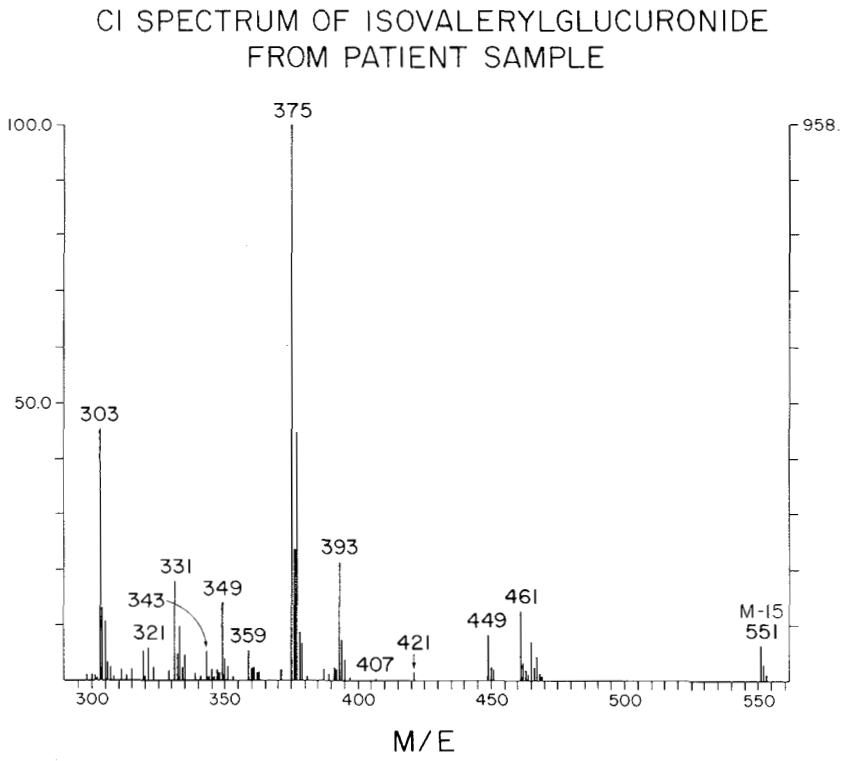

Fig. 3. Chemical ionization mass spectrum of gas chromatography peak #11 from Figure 1.

in our organic acid TMS method. Significant amounts of isovaleryl glucuronide (20–30%) were detected in the lyophilized aqueous phase after four extractions with ethyl acetate.

Some investigators have questioned the selectivity of β-glucuronidase and have speculated that the enzyme may also cleave glycine conjugates (3). In order to rule out isovalerylglycine as the source of the isovaleric acid liberated by glucuronidase treatment of the urine sample, we incubated authentic isovalerylglycine (1 μmol/ml) with the same β-glucuronidase preparation. We detected no release of free isovaleric acid, excluding hydrolysis of isovalerylglycine by this enzyme.

In order to identify glucuronic acid in the β-glucuronidase-treated urine, we acidified the urine sample and extracted several times with ethyl acetate. Glucuronic acid is insoluble in organic

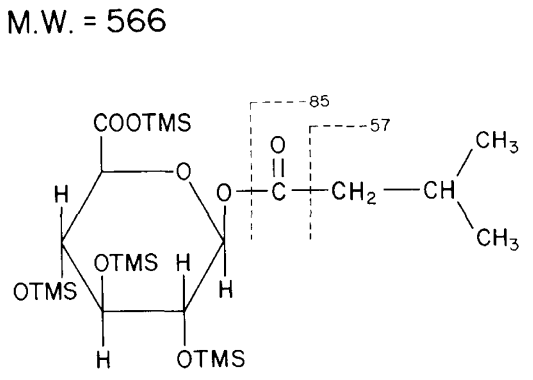

Fig. 4. Structure of isovaleryl glucuronide tetra-TMS.

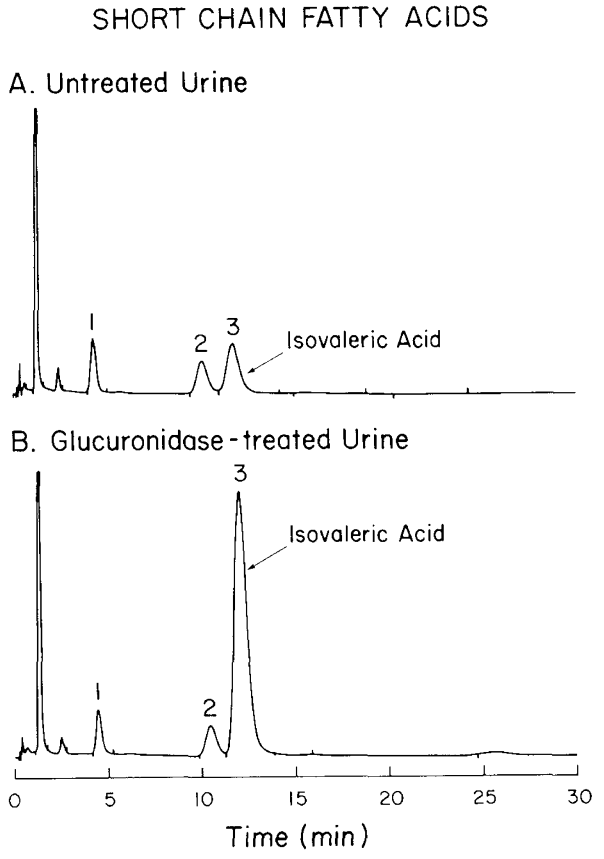

Fig. 5. Short chain fatty acid gas chromatograms (GC): *A*) an isovaleric acidemia urine sample treated with buffer alone and *B*) the same isovaleric acidemia urine sample treated with *E. coli* β-glucuronidase. The numbered GC peaks were identified as: *1*) isobutyric acid; *2*) 2-methylbutyric acid; and *3*) isovaleric acid.

solvents and remained in the water layer. The water layer was lyophilized and the dried residue was silylated with Tri-Sil BSA. A well-defined peak which elutes before the glucuronide conjugate (methylene unit 20.88, 3% OV-1) was positively identified as glucuronic acid penta-TMS by GC-MS by matching the spectrum obtained to that of authentic glucuronic acid penta-TMS. The peak obtained was smaller than might have been expected. We also carried out a similar experiment using authentic phenolphthalein glucuronide. The recovery of glucuronic acid from the phenolphthalein glucuronide hydrolyzate by the same β-glucuronidase was similar to that observed in the experiment using the urine of patients with isovaleric acidemia. The failure of quantitative recovery of glucuronic acid may be due partly to the fact that glucuronic acid also forms various lactones (2).

Urinary excretion pattern. We have identified isovaleryl glucuronide in the urine of patients with isovaleric acidemia. This is the first time that detoxification via glucuronide conjugation has been demonstrated in an inborn error of metabolism. The glucuronide conjugation of isovaleric acid is probably an important aspect of the detoxification mechanisms in isovaleric acidemia.

In order to ascertain the precipitating factors which lead to the excretion of isovaleryl glucuronide, we reviewed 72 chromatograms from four patients with isovaleric acidemia. We suspected that glucuronide conjugation may become activated when the glycine conjugation system is saturated. When the glycine conjugation system is saturated, free isovaleric acid is oxidized to 3-hydroxyisovaleric acid, which appears in the urine (10). We examined the excretion pattern of 3-hydroxyisovaleric acid, isovaleryl glucuronide, and isovalerylglycine as shown in Tables 1 and 2. The peaks for the three metabolites were identified using methylene unit valves compiled on 10% OV-1 and 10% OV-17 packed columns (12). The homogeneity of the gas chromatogram

Table 1. *Urinary excretion of isovaleryl glucuronide and isovalerylglycine as related to the amount of 3-hydroxyisovaleric acid (values expressed as mg/mg creatinine)*

3-Hydroxyisovaleric acid	Isovaleryl glucuronide	Isovaleryglycine
0 ($n = 49$)*	0 ($n = 31$)–0.54	1.07–12.27
0.04	0	2.77
0.04	0	6.72
0.05	0	3.88
0.07	0	3.80
0.07	0.04	4.70
0.07	0	5.51
0.07	0.03	6.29
0.08	0.04	1.56
0.08	0	3.11
0.08	0	5.02
0.08	0	5.51
0.09	0.08	3.31
0.16	0.45	5.28
0.26	0	5.87
0.26	0	11.90
0.30	0.02	5.02
0.34	0	6.60
0.38	0.05	1.07
0.39	0.04	5.49
0.77	0.07	8.46
0.88	0.23	5.84
0.90	0.25	5.70
1.00	0.10	7.74

* The number of samples are indicated in parenthesis.

Table 2. *The amounts of 3-hydroxyisovaleric acid and isovalerylglycine in urine samples containing, and not containing, isovalerylglucuronide*

	Number of samples	3-Hydroxyisovaleric acid* (mg/mg creatinine)	Isovalerylglycine (mg/mg creatinine)
Samples containing isovaleryl glucuronide	29	0.176 ± 0.312†	4.38 ± 1.90
Samples containing no isovaleryl glucuronide	43	0.032 ± 0.076	4.70 ± 2.62

* Twenty-three samples contained 3-hydroxyisovaleric acid.
† Values given as mean ± 1 SD.

peaks was confirmed, in each case, for five randomly selected samples which were analyzed by GC-MS. Although there is no linear, quantitative correlation between the amounts of 3-hydroxyisovaleric acid and isovaleryl glucuronide, it is apparent that isovaleryl glucuronide is more likely to be excreted when the amount of 3-hydroxyisovaleric acid is high. For example, as can be seen in Table 1, when the amount of 3-hydroxyisovaleric acid was greater than 0.26 mg/mg creatinine, isovaleryl glucuronide was present in seven out of eight samples. When the amount of 3-hydroxyisovaleric acid was equal to or less than 0.26 mg/mg creatinine, isovaleryl glucuronide was present in only 23 of 64 samples. Table 2 shows that the average amount of 3-hydroxyisovaleric acid was more than five times greater in those samples that contained isovaleryl glucuronide, whereas the amount of isovalerylglycine was roughly equal in both groups. The data therefore does indicate that glucuronide conjugation of isovaleric acid is more prevalent when the glycine conjugation system is saturated. It should be noted, however, that of the 49 samples which contained no measurable amount of 3-hydroxyisovaleric acid, 18 contained isovaleryl glucuronide in varying amounts ranging up to 0.54 mg/mg creatinine (see Table 1). There may be other factors involved in the stimulation of the glucuronide detoxification mechanism. Nevertheless, glucuronide conjugation appears to be assuming the role of a secondary, yet important, detoxification mechanism in isovaleric acidemia.

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Colostrum-Induced Enteric Mucosal Growth in Beagle Puppies

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Summary

To evaluate the role of artificial feeding and natural feeding in early growth of enteric mucosa, we determined enteric mucosal mass, protein and DNA content, and disaccharidase activities in beagle puppies at birth, and after 24 h of either natural or artificial feeding. Despite similar increases in body weight over the first 24 h of life, neither mucosal mass, DNA content, nor protein content of the artificially fed animals was different from that of newborn animals. In contrast, mucosal mass of the suckled animals was 75% greater, DNA content was 56% greater, and protein content was 93% greater than that of newborn animals. The mucosal protein/DNA ratio of the suckled animals was greater than that of newborn, but not artifically fed animals. The greater DNA, protein, and protein/DNA ratio in this group suggest that the greater mucosal mass is a result of both cellular hyperplasia and hypertrophy. Sucrase specific activity of the suckled animals was less than that of the artificially fed but not the newborn animals. Other disaccharidase activities were not different among the three groups.

These data extend the findings of Widdowson *et al.* (25) to another species and demonstrate that this rapid enteric growth over the first day of life results only from natural feeding. They strongly suggest, therefore, that rapid early enteric growth, mediated perhaps by a factor in natural milk that stimulates enteric mucosal growth, is an important heretofore unappreciated phase of intestinal development.

In contrast to the well described alterations that take place immediately after birth in many organ systems (i.e., the cardiovascular and respiratory symptoms), very little is known about