Abstract
Breastfeeding induces a different metabolic and endocrine response than feeding conventional infant formula, and it has also been associated with slower weight gain and reduced disease risk in later life. The underlying programming mechanisms remain to be explored. Breastfeeding has been reported to induce lower levels of insulin, insulin-like growth factor-1 and some amino acids (AAs) than formula feeding. In the Childhood Obesity Project (CHOP), infants fed a conventional protein-rich formula had a higher BMI at 2 and 6 years than those fed a protein-reduced formula. At 6 months, higher protein intakes induced increased plasma concentrations of branched-chain AAs (BCAAs) and their oxidation products, short-chain acylcarnitines. With increasing BCAA levels, these short-chain acylcarnitines increased proportionally only until a break point was reached, after which BCAAs seemed to escape their degradation. The resulting marked elevation in BCAA levels with high-protein (HP) intakes appears to contribute to increased insulin levels and to affect β-oxidation of fatty acids. The ratios of long-chain acylcarnitines to free carnitine decreased in infants who received a HP formula, which indicates a reduced initiation of β-oxidation. We conclude that HP intakes inducing high BCAA plasma levels may inhibit fat oxidation and thereby enhance body fat deposition and adiposity.
**Introduction**

The infant’s metabolic response adapts to environmental and particularly dietary exposure, and appears to affect growth, body composition and later disease risks [1]. Compared to feeding a conventional infant formula, breastfeeding was shown to induce different metabolic and endocrine responses, and it has been associated with differences in growth, body composition and disease risk throughout childhood and in adult life [2]. Populations of breastfed (BF) children show a lower prevalence of overweight and obesity at school age. The ‘Early Protein Hypothesis’ links the amount of protein in infant feeding to weight gain in the first months of life, which is related to obesity risk in childhood and early adulthood [3]. The underlying programming mechanisms remain to be explored. Recent publications point to epigenetic modifications of genes affecting different proteins and hormones, like leptin, insulin-like growth factor (IGF)-1 and insulin [4]. Associations of endocrine and hormonal markers with obesity are discussed by Socha et al. [this vol., pp. 81–88].

**Effect of the Infant Formula on the Metabolome**

Regardless of the primary mechanism, changes will be induced in the metabolome of infants fed different diets, since metabolites (molecules <1,500 Da) are the downstream products of both genetic and epigenetic alterations as well as environmental factors, including diet. The metabolome is closely related to the phenotype. More important, metabolomics is capable to enhance the understanding of metabolic regulation in response to environmental influences [5]. It is expected that the ‘programming effects’ of infant formula with different contents of protein should be reflected in the metabolome and consequently in the regulation of metabolic pathways. Martin et al. [6] found differences in the metabolism of formula-fed (FF) babies from obese mothers compared to BF infants. Higher short-chain acylcarnitines C2, C3, C4 and amino acids (AAs) were found in stool of FF infants suggesting an increased breakdown of protein in the gut by bacteria. Differences in the urinary metabolome pointed to an increased protein metabolism in FF infants. Furthermore, β-oxidation and ketogenesis were affected by formula feeding. In 1998, Karlsland Akeson et al. [7] reported increased values of some essential AAs in blood plasma of healthy infants at the age of 6 months, who received exclusive breastfeeding until the age of 3 months and afterwards were randomly assigned to infant formulae with either 13, 15 or 18 g/l protein (F13, F15, F18 in table 1). Since the sample number was very small and the mothers were allowed to breastfeed as long as they wished and to intro-
duce the assigned formulas gradually, the effect of the formula intake was van-
ished. In the BeMIM study, infants of mothers who chose formula feeding re-
ceived either a standard formula (2.2 g/100 kcal protein) or an intervention for-
mula with lower protein (LP) content (1.89 g/100 kcal protein) and modified
protein composition, which was introduced within 28 days after birth [8]. The
intervention formula contained additional α-lactalbumin, free phenylalanine,
free tryptophan and long-chain polyunsaturated fatty acids. A BF group was also
followed for reference. Urea and AA levels, in particular nondispensable AA
levels, were higher in the blood plasma of both groups of FF infants (table 1).
Nonessential or dispensable AAs were less affected by the different diets or even
decreased in the plasma of FF infants, e.g. glutamine, glutamate or serine.

### Table 1. Plasma concentrations of AAs (μM) in FF and BF infants in three different studies

<table>
<thead>
<tr>
<th>Formula</th>
<th>CHOP [11, 12], 6 months</th>
<th>BeMIM [8], 4 months</th>
<th>Karlsland Akeson et al. [7], 6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LP</td>
<td>HP</td>
<td>BF</td>
</tr>
<tr>
<td>Protein, g/100 kcal</td>
<td>1.8</td>
<td>2.9</td>
<td>–</td>
</tr>
<tr>
<td>Sample size, n</td>
<td>260</td>
<td>262</td>
<td>158</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>64</td>
<td>85*</td>
<td>58</td>
</tr>
<tr>
<td>Leucine</td>
<td>120</td>
<td>165*</td>
<td>106</td>
</tr>
<tr>
<td>Lysine</td>
<td>166</td>
<td>197*</td>
<td>145</td>
</tr>
<tr>
<td>Methionine</td>
<td>31</td>
<td>35*</td>
<td>24</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>72*</td>
<td>84*</td>
<td>61</td>
</tr>
<tr>
<td>Threonine</td>
<td>126</td>
<td>142*</td>
<td>119</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>56</td>
<td>67</td>
<td>60</td>
</tr>
<tr>
<td>Valine</td>
<td>214*</td>
<td>304*</td>
<td>172</td>
</tr>
<tr>
<td>Alanine</td>
<td>440</td>
<td>420</td>
<td>430</td>
</tr>
<tr>
<td>Arginine</td>
<td>115</td>
<td>110</td>
<td>113</td>
</tr>
<tr>
<td>Asparagine</td>
<td>54</td>
<td>58</td>
<td>52</td>
</tr>
<tr>
<td>Aspartate</td>
<td>25</td>
<td>27</td>
<td>26</td>
</tr>
<tr>
<td>Glutamine</td>
<td>605*</td>
<td>556*</td>
<td>664</td>
</tr>
<tr>
<td>Glutamate</td>
<td>122</td>
<td>115</td>
<td>130</td>
</tr>
<tr>
<td>Glycine</td>
<td>267*</td>
<td>230</td>
<td>220</td>
</tr>
<tr>
<td>Histidine</td>
<td>105*</td>
<td>107*</td>
<td>88</td>
</tr>
<tr>
<td>Serine</td>
<td>161*</td>
<td>159*</td>
<td>187</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>83*</td>
<td>101*</td>
<td>66</td>
</tr>
<tr>
<td>Citrulline</td>
<td>32*</td>
<td>34*</td>
<td>27</td>
</tr>
<tr>
<td>Ornithine</td>
<td>116</td>
<td>116</td>
<td>121</td>
</tr>
<tr>
<td>Proline</td>
<td>316</td>
<td>365*</td>
<td>319</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. BF. p values were obtained by a linear mixed model adjusted for study center and corrected for multiple testing (CHOP); by Kruskal-Wallis tests (BeMIM), and by nonparametric Kruskal-Wallis and Mann-Whitney tests and analyses of variance, using the post hoc test of Bonferroni/Dunn (Karlsland Akeson et al. [7]).
Childhood Obesity Project – Lower versus Higher Protein Intake

In a large, double-blind, randomized, clinical intervention trial, we studied the effect of infant and follow-on formula with conventionally higher protein (HP, 2.05 g/100 ml protein) or LP (1.25 g/100 ml protein) contents on infant growth and metabolic responses. In this trial, the HP formula led to higher BMI than the LP formula at 2 years of age [9] and at school age [10]. Total IGF-1 serum levels were increased in the HP group, whereas IGF-binding protein (IGFBP)-2 was lower and IGFBP-3 did not differ significantly between both formula groups at the age of 6 months [11].

At the age of 6 months, HP-fed children showed significantly increased plasma concentrations of nondispensable AAs (table 1), including the branched-chain amino acids (BCAAs; Ile, Leu and Val) [11] as well as increased levels of the oxidation products of BCAAs (short-chain acylcarnitines) compared to LP intake as well as to a reference group of BF infants (table 2) [12]. Also, urea increased significantly in both the LP and HP groups compared to the BF group.

Branched-Chain Amino-Acid Metabolism in Formula-Fed Infants

Given that elevated levels of BCAAs and their degradation products are associated with infant formula intake, the BCAA metabolism might be the potential key factor in the relation between formula feeding and later obesity development. BCAAs are less metabolized during the first pass in the liver compared to other AAs [13]. In general, dietary proteins are degraded in the intestine to peptides and free AAs, which are resorbed. After intestinal resorption and metabolism, the portal vein transports the AAs to the liver where they undergo first-pass metabolism. However, the key enzyme of BCAA oxidation, branched-chain α-keto acid dehydrogenase (BCKDH), is less present in the liver [13]. Thus, the BCAA output of the liver is enhanced compared to other AAs, and BCAAs are much more increased in the plasma of HP-fed children than other essential AAs.

In the skeletal muscle, BCAAs are degraded for energy provision [14]. First, valine, leucine and isoleucine are transaminased by the branched-chain amino transferase to α-keto acids (fig. 1). These keto acids are subsequently reduced by BCKDH to short-chain fatty acids, which are bound to short-chain acylcarnitines C4 and C5 [15]. Further degradation products comprise the acylcarnitine C3, C5-OH and C5:1. The reduction step via BCKDH is the limiting factor in the degradation of BCAAs [13]. Leucine supplementation increases BCKDH activity [14] to ensure higher degradation of BCAAs in a state of high BCAA availability to keep BCAA levels in a physiological range. In infants in the Childhood
### Table 2. Means (SD) of plasma concentrations (μM) of short-chain acylcarnitines (Carn) in HP- and LP-fed infants participating in the CHOP trial

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>LP (n = 260)</th>
<th>HP (n = 262)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Carn</td>
<td>38 (7.05)</td>
<td>40 (7.32)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Carn C2</td>
<td>5.4 (2.35)</td>
<td>4.8 (2.34)</td>
<td>0.14</td>
</tr>
<tr>
<td>Carn C3</td>
<td>313 × 10⁻³ (0.1)</td>
<td>479 × 10⁻³ (0.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Carn C4-OH</td>
<td>72 × 10⁻³ (0.03)</td>
<td>67 × 10⁻³ (0.05)</td>
<td>1</td>
</tr>
<tr>
<td>Carn C3-OH</td>
<td>23 × 10⁻³ (0.004)</td>
<td>23 × 10⁻³ (0.004)</td>
<td>1</td>
</tr>
<tr>
<td>Carn C3:1</td>
<td>6.7 × 10⁻³ (0.002)</td>
<td>6.5 × 10⁻³ (0.002)</td>
<td>1</td>
</tr>
<tr>
<td>Carn C4</td>
<td>128 × 10⁻³ (0.05)</td>
<td>206 × 10⁻³ (0.09)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Carn C4:1</td>
<td>12 × 10⁻³ (0.002)</td>
<td>12 × 10⁻³ (0.002)</td>
<td>1</td>
</tr>
<tr>
<td>Carn C5</td>
<td>95 × 10⁻³ (0.04)</td>
<td>154 × 10⁻³ (0.06)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Carn C5-M-DC</td>
<td>41 × 10⁻³ (0.006)</td>
<td>39 × 10⁻³ (0.006)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Carn C5-OH</td>
<td>39 × 10⁻³ (0.009)</td>
<td>45 × 10⁻³ (0.01)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Carn C5:1</td>
<td>18 × 10⁻³ (0.007)</td>
<td>21 × 10⁻³ (0.008)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Carn C5:1-DC</td>
<td>19 × 10⁻³ (0.009)</td>
<td>18 × 10⁻³ (0.01)</td>
<td>1</td>
</tr>
<tr>
<td>Carn C5-DC</td>
<td>25 × 10⁻³ (0.008)</td>
<td>20 × 10⁻³ (0.007)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

p values were obtained by a linear mixed model adjusted for study center and corrected for multiple testing (adapted from Kirchberg et al. [12]).

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**Fig. 1.** Degradation pathway of BCAAs. Leucine activates the rate-limiting enzyme BCKDH. The occurring short-chain acyl chains are bound to free carnitine. oxo = Molecule contains a keto group; OH = molecule contains a hydroxyl group; DC = molecule contains two carboxyl groups.
Obesity Project (CHOP), the European CHOP Trial Study Group (see Appendix) demonstrated a saturation of this pathway. Segmented regression models revealed that with increasing BCAA levels, the short-chain acylcarnitines only increased until a break point was reached (fig. 2) [12]. After this point, the corresponding short-chain acylcarnitines C4 and C5 did not longer increase with

Fig. 2. The relation between BCAAs (Ile, Leu and Val) and their corresponding short-chain acylcarnitine in high-protein fed infants indicates a concentration-dependent saturation of BCAA catabolism in infants. Modified from Kirchberg et al. [12].
increasing BCAA concentration. Thus, BCAAs seemed to escape their degrada-
tion after a certain point of high plasma BCAA levels, which indicates a satura-
tion of BCAA catabolism in infants. This was especially observed for the HP
group, who reached higher plasma levels of BCAAs. To our knowledge, this is
the first indication that above a certain plasma concentration, the BCAA degra-
dation pathway becomes saturated. This could potentially be of major biological
importance in infants fed a high amount of protein, and where a markedly in-
creased risk of adverse effects mediated through BCAAs may result. Leucine, for
instance, is a potent stimulator of insulin secretion [16]. Increased C-peptide/
creatinine ratios in HP fed infants were shown in the CHOP trial [6].

Furthermore, leucine depressed β-oxidation of fatty acids [17]. The ratio of
long-chain acylcarnitines to free carnitine decreased in infants who received HP
formula in the CHOP trial, which indicates a lower initial step of the β-oxidation
(table 3) [12]. Moreover, leucine deprivation resulted in reduced activity of fatty
synthase genes [18]. This deregulation of fat metabolism may result in a lipid
oversupply, which causes consequently lipotoxicity, insulin resistance and fat
storage [19]. Thus, HP and BCAA intake may inhibit fat oxidation and thereby
enhance body fat deposition and the risk of adiposity. This would explain the
effects of HP feeding on increased weight gain during the first years of live. The
absence of significant differences for leucine and isoleucine as well as acylcarni-
tines C4 and C5 between LP-fed and BF infants underline the influence of HP-
feeding on the metabolism.

**Other Indispensable Amino Acids**

Infant protein supply also affects the metabolism of other AAs. Levels of aro-
matic AAs (AAAs), particularly phenylalanine, which promotes IGF-1 secre-
tion, were also elevated in the plasma of conventional FF infants (table 1). Levels

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**Table 3.** Means (SD) of ratios of the long-chain acylcarnitines C14, C16 and C18 to free
carnitine (Carn) in HP- and LP-fed infants participating in the CHOP trial

<table>
<thead>
<tr>
<th>Ratios</th>
<th>LP (n = 260)</th>
<th>HP (n = 262)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14/free Carn</td>
<td>1.2 × 10⁻³ (0.0003)</td>
<td>1.0 × 10⁻³ (0.0004)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C16/free Carn</td>
<td>2.6 × 10⁻³ (0.0007)</td>
<td>2.2 × 10⁻³ (0.0008)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C18/free Carn</td>
<td>0.8 × 10⁻³ (0.0002)</td>
<td>0.7 × 10⁻³ (0.0002)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

p values were obtained from a linear mixed model adjusted for study center and correct-
ed for multiple testing (adapted from Kirchberg et al. [12]).
of the nonessential AAA tyrosine are also increased in the HP group due to transformation of phenylalanine to tyrosine [20]. The trials showed that HP diet resulted in higher levels of AAAs compared to LP diet (table 1). BCAAs and AAAs compete for transportation in mammalian tissues [21]. Therefore, higher values of BCAAs result in a lower uptake of AAAs, e.g. in the brain, and higher AAA plasma levels [22]. Reduction in AAA levels in the brain lower the synthesis and the release of neurotransmitters like serotonin and catecholamines. This affects metabolic pathways. As BCAAs, AAAs are insulinogenic [16] and elevated levels are related to obesity and may predict future diabetes [23]. Regarding the effect of AAAs on IGF-1 levels, AAAs may represent the missing link between HP intake or leucine supplementation and elevated IGF-1 levels [24]. In conclusion, not only the amount of protein in formula, but also the composition and the kind of the protein used are related to the later adverse outcomes in FF infants.

Nearly all other essential AAs were elevated in FF infants and were particularly strongly affected by HP diet (table 1). Higher concentrations of certain essential AAs, namely leucine, phenylalanine, tyrosine and lysine, are well known to contribute to an elevated insulin secretion [16]. In contrast, a contributory effect of elevated levels of essential AAs on growth hormone and IGF-1 levels may be assumed, but further investigations in human intervention studies are needed to get a more detailed picture of the underlying molecular mechanisms. However, elevated essential AAs and their effect on the secretion of growth factors may be an underlying mechanism of the Early Protein Hypothesis [3].

Dispensable Amino Acids – The Decrease in Glutamine

Nonessential AAs are less affected by HP diet than indispensable AAs. The lesser influence of the diet on nonessential AAs appears to be due to regulating mechanisms. Since these AAs are endogenously synthesized, the human metabolism can downregulate the biosynthesis of these AAs in times of protein oversupply to keep levels in the tissues and the blood plasma constant. This regulation mainly appears in the intestine and the liver during the first pass, hence, in contrast to oral supplements, direct infusion may affect plasma levels. Not in accord with this hypothesis, glutamine levels are decreased in different studies investigating FF infants (table 1). In the CHOP trial, glutamine was lower in HP-fed infants compared to LP-fed infants, and even the LP-fed infants had lower levels compared to BF infants. An alteration in the urea cycle is assumed, because urea is elevated in FF infants [8, 11, 25]. However, levels of other AAs involved in the urea cycle, namely glutamic acid, aspartic acid, arginine and or-
nithine, showed no consistent picture or were not affected by formula diet (table 1), whereas citrulline was elevated in the CHOP and the BeMIM trial. In contrast to glutamine, cells can recycle the other AAs during the urea or the aspartate cycle [26]. Glutamic acid can be recycled at the expense of glutamine. Thus, glutamine may be the only AA with decreased levels by an elevated urea cycle. Elevation in the urea cycle in the formula groups could result from enhanced protein intake and the subsequent higher protein metabolism. Another explanation of the lower glutamine levels in the HP group might be the contribution of glutamine to insulin secretion induced by leucine [27]. Leucine activates glutamate dehydrogenase in pancreatic islets resulting in consumption of glutamic acid. In pancreatic islets, glutamic acid is mainly provided by the intracellular conversion of glutamine to glutamic acid [28]. Hence, increased insulin release, enhanced by leucine levels, may decrease glutamine levels.

One Step Further – The Link to Early Weight Gain and Obesity Risk

In infants, metabolites that responded to HP supply have been previously reported as markers for obesity risk. BCAAs, nonesterified fatty acids, organic acids, acylcarnitines and phospholipids were identified as potential biomarkers for obesity in a recent review [29]. This indicates a relation of elevated BCAAs by HP diet to the obese state [21, 23]. Furthermore, a deregulation of the β-oxidation seems to be associated with the development of obesity and insulin resistance. Nevertheless, the underlying mechanisms and pathways require further exploration. The CHOP trial offers the possibility to analyze the onset of obesity and the change in metabolites over the period of obesity development longitudinally. For instance, it was shown in the CHOP trial that lysophosphatidylcholine 14:0 is strongly related to rapid weight gain in infancy in the first 6 months of life and to overweight/obesity at the age of 6 years [30]. However, unraveling the effects of infant formula on the metabolome remains challenging, and further trials will provide insights in the molecular mechanism and help to optimize infant formulas. Metabolites like keto acids, or intermediates of the citric acid cycle or from gluconeogenesis, should be analyzed in response to formula feeding and may give new insights in the future.

Conclusion

HP intake in excess of metabolic requirements increases BCAA concentrations in infant plasma to levels at which the normal catabolic capacity for BCAAs is exceeded. Thereby, high dietary protein supply to infants may stimulate mark-
edly enhanced secretion of the growth factors insulin and IGF-1, and induce signaling effects inducing excessive weight gain. Moreover, HP intake in infants appears to inhibit initiation of β-oxidation and thus may contribute to enhanced fat storage and increased adiposity, probably by enhanced BCAA levels. Elevated levels of BCAAs and disturbed β-oxidation have been shown in previous observational studies to be associated with obesity and risk of cardiovascular disease. Thus, BCAA metabolism might present a mechanism linking infant formula feeding and obesity risk.

Appendix

The European CHOP Study Group
Philippe Goyens, Clotilde Carlier, Pascale Poncelet, Elena Dain and Joana Hoyos (Free University of Brussels, Brussels, Belgium); Françoise Martin, Annick Xhonneux, Jean-Paul Langhendries and Jean-Noel Van Hees (Centre Hospitalier Chrétien St Vincent, Liège, Belgium); Ricardo Closa-Monasterolo, Joaquin Escribano, Veronica Luque, Georgina Mendez, Natalia Ferre and Marta Zaragoza-Jordana (Universitat Rovira I Virgili, Tarragona, Spain); Marcello Giovannini, Enrica Riva, Carlo Agostoni, Silvia Scaglioni, Elvira Verduci, Fiammetta Vecchi and Alice Re Dionigi (University of Milan, Milan, Italy); Jerzy Socha, Anna Dobrzańska, Dariusz Gruszfeld, Piotr Socha, Anna Stolarczyk, Agnieszka Kowalik, Roman Janas and Ewa Pietraszek (Children’s Memorial Health Institute, Warsaw, Poland); Emmanuel Perrin (Danone Research Center for Specialized Nutrition, Schiphol, The Netherlands); Helfried Groebe, Anna Reith, and Renate Hofmann (Klinikum Nurnberg Sued, Nurnberg, Germany); Berthold Koletzko, Veit Grote, Martina Weber, Sonia Schiess, Jeannette Beyer, Michaela Fritsch, Uschi Handel, Ingrid Pawellek, Sabine Verwied-Jorky, Iris Hannibal, Hans Demmelmaier, Gundrun Haile, Wolfgang Peissner, Ulrike Harder, Franca F. Kirchberg, Melissa Theurich, Peter Rzehak, Christian Hellmuth and Olaf Uhl (Dr. von Hauner Children’s Hospital, Ludwig Maximilian University of Munich, Munich Germany), and Rüdiger von Kries (Institute for Social Pediatrics and Adolescent Medicine, University of Munich, Munich, Germany).

Acknowledgment

This work was financially supported by the Commission of the European Communities, the seventh Framework Program, contract FP7-289346-EarlyNutrition, and the European Research Council Advanced Grant ERC-2012-AdG (No. 322605 META-GROWTH). This paper does not necessarily reflect the views of the Commission and in no way anticipates the future policy in this area. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the paper. Funds to support the writing of the paper were provided by Nestlé Nutrition, Vevey, Switzerland.
Disclosure Statement

C.H. received funds by Nestle Nutrition, Vevey, Switzerland to support the preparation of the paper. C.H. and P.S. presented the work at the corresponding Nestle Nutrition workshop. All other authors have nothing to disclose.

References


