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Anthocyanins: Structural characteristics that result in unique metabolic patterns and biological activities*

RONALD L. PRIOR[1,2] & XIANLI WU[2]

[1]*US Department of Agriculture, Agriculture Research Service, Arkansas Children's Nutrition Center, 1120 Marshall Street, Little Rock, AR 72202, USA, and* [2]*Arkansas Children's Nutrition Center, 1120 Marshall Street, Little Rock, AR 72202, USA*

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Abstract
Interest in anthocyanins has increased immensely during the past decade. From these studies, it is clear that anthocyanins have unique properties: Anthocyanins are absorbed intact and absorption can be saturated; acylation of anthocyanins lowers their apparent absorption; anthocyanidin diglycosides in the form of sambubioside or rutinoside impart increased stability to the anthocyanin molecule; and the quantities excreted in urine are less than 0.1% of intake. However, 60–90% of the anthocyanins may disappear from the gastrointestinal tract within 4 h after a meal. What happens to the bulk of the anthocyanins that disappear is not clear. Degradation accounts for a part of this disappearance, but differs for the various aglycones and may be modified further by the nature of the aglycone glycosylation, which further complicates our understanding of this process. Anthocyanins may play an important role in health promotion in terms of obesity prevention, cardiovascular health, anti-inflammatory and anti-cancer effects.

Keywords: *Anthocyanins, antioxidant, absorption, brain, obesity, metabolism*

Abbreviations: *Glc, glucoside; gal, galactoside; arab, arabinoside; rut, rutinoside; samb, sambubuioside; xyl, xyloside; Cy, cyanidin; Dp, delphinidin; Pn, peonidin; Pt, Petunidin; Mv, Mavidin*

Introduction

As a major sub-group of flavonoids, anthocyanins are water soluble plant pigments responsible for the blue, purple and red color of many plant tissues. They occur primarily as glycosides of their respective aglycone anthocyanidin-chromophores (Figure 1), with the sugar moiety mainly attached at the 3-position on the C-ring or the 5, 7-position on the A-ring. Glycosylation at the 3′-, 4′-, 5′-positions of the B-ring, although very rare, has also been observed [1]. Glucose (glc), galactose (gal), arabinose (arab), rhamnose (rham) and xylose (xyl) are the most common sugars that are bonded to anthocyanidins as mono-, di- or trisaccharide forms. Except for the 3-deoxyanthocyanidins such as luteolinidin and apigeninidin in sorghum [2], aglycones are rarely found in fresh plant materials. There are about 17 anthocyanidins found in nature, whereas only six of them, cyanidin (Cy), delphinidin (Dp), petunidin (Pt), peonidin (Pn), pelargonidin (Pg) and malvidin (Mv), are ubiquitously distributed (Figure 1).

The differences in chemical structure of these six common anthocyanidins occur at the 3′ and 5′ positions of the B-ring (Figure 1). The sugar moieties

Correspondence: R. L. Prior, USDA, Arkansas Children's Nutrition Center, 1120 Marshall Street, Little Rock, AR 72202, USA. Tel: 1 501 354 2747. Fax: 1 501 364 2818. E-mail: priorronaldl@uams.edu

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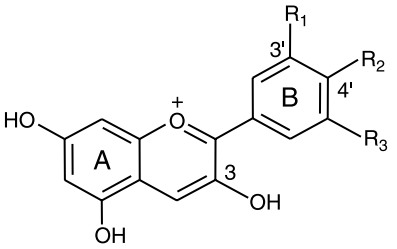


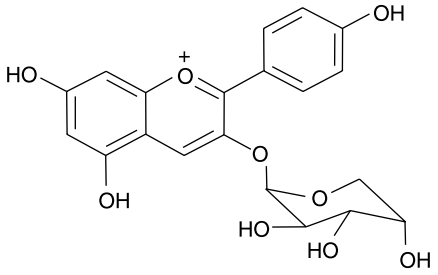
ANTHOCYANIDIN	R_1	R_2	R_3
Pelargonidin (Pg)	H	OH	H
Cyanidin (Cy)	OH	OH	H
Delphinidin (Dp)	OH	OH	OH
Peonidin (Pn)	OMe	OH	H
Petunidin (Pt)	OMe	OH	OH
Malvidin (Mv)	OMe	OH	OMe

Figure 1. Chemical structures of six common anthocyanidins.

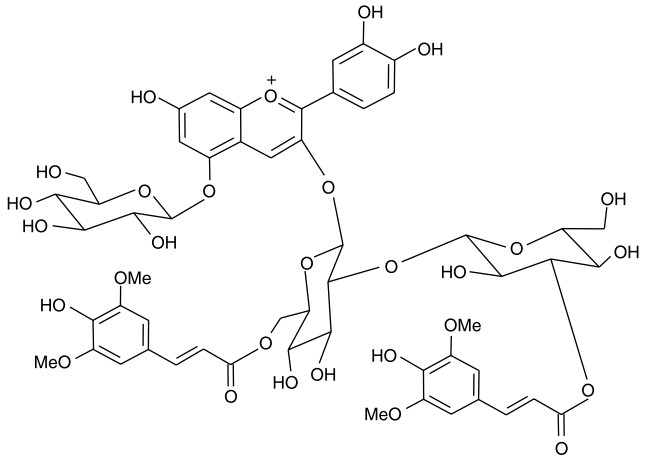
may also be acylated by a range of aromatic or aliphatic acids (Figure 2). Common acylating agents are cinnamic acids. Over 600 naturally occurring anthocyanins have been reported [3] and they are known to vary in: (1) the number and position of hydroxyl and methoxyl groups on the basic anthocyanidin skeleton; (2) the identity, number and positions at which sugars are attached; and (3) the extent of sugar acylation and the identity of the acylating agent [4]. Thus, the chemical structures of anthocyanins vary significantly depending on the extent of glycosylation and acylation (Figure 2). For most anthocyanins, their molecular weights range from 400 to 1200. An example of some of the complex structures that can occur in foods is illustrated in Figure 2 and in publications by Wu et al. [2,5].

Unlike other sub-groups of flavonoids with the same C6–C3–C6 skeleton, anthocyanins have a positive charge in their structure at acidic pH. In solution, the anthocyanin actually occurs in equilibrium with essentially four molecular forms: the flavylium cation, the quinoidal base, the hemiacetal base and chalcone [6]. The relative amounts of these four forms vary with both pH and structure of the anthocyanins [1,7,8]. Anthocyanins exist primarily as the stable flavylium cation only when the pH < 2. This uniqueness in the chemical structure is one of the key factors that affect the absorption, metabolism, bioavailability and consequently, the biological responses to anthocyanins.

Anthocyanins have been shown to be strong antioxidants, and may exert a wide range of health benefits through antioxidant or other mechanisms [4]. However, without knowing the rate and extent of their absorption, metabolism and tissue or cell distribution, the role of anthocyanins in disease prevention will still be an enigma. Furthermore, for



Pelargonidin 3-arabinoside (MW = 403) (From Strawberry)



Cyanidin 3-(sinapoyl)diglucoside-5-(sinapoyl)glucoside (MW = 1185) (From Red Cabbage)

Figure 2. Chemical structures of two anthocyanins identified from strawberry or red cabbage.

some compounds including anthocyanins, their primary forms found circulating in blood or in tissues after oral ingestion may not necessarily be the original forms in the diet, but are the metabolites or breakdown compounds generated during absorption and/or metabolism. These newly generated compounds may or may not have the same biological effects compared to their precursors. Thus, knowledge about the absorption, metabolism and bioavailability of anthocyanins is a crucial step in order to understand their health effects.

Excretion of pigmented urine from rabbits fed grapes was observed and published as early as 1933 [9], however, the study of anthocyanin bioavailability did not really begin until the novel findings that anthocyanins were absorbed intact without deglycosylation in the late 20th century [10–12]. Since then, some 20 studies have been conducted with anthocyanins in different food sources or extracts in the rat, rabbit, pig or human (See [4,13,14] for reviews). Some excellent reviews regarding bioavailability of flavonoids or polyphenols have dealt with this topic [4,13–19]. However, in this review, we will focus exclusively on anthocyanins and summarize primarily progress achieved during the last 2–3 years.

Anthocyanin absorption

Results of absorption of anthocyanins from different studies have not always agreed. From our current knowledge, at least five major characteristics have been recognized to be related to absorption of anthocyanins. These characteristics make anthocyanins very different from other flavonoids or polyphenolic compounds.

Anthocyanins can be absorbed intact as glycosides

Due to the high polarity, flavonoid glycosides were generally considered too hydrophilic for absorption by passive diffusion in the small intestine [14,20]. In order to be absorbed, they need either a specific transporter, such as the intestinal Na^+-dependant glc co-transporter (SGLT1) [21], to transport the glucoside across the small intestine; or they have to be hydrolyzed to the aglycone forms by enzymes (eg. lactase phloridzin hydrolase, LPH) [22,23] or by colonic bacteria [24,25] prior to absorption. However, anthocyanins have been detected in both plasma and urine as intact glycosides [4]. The organic anion carrier bilitranslocase also has been proposed as a carrier protein for anthocyanin absorption because its transport activity is competitively inhibited by anthocyanins [26] and by two different specific antibodies [26]. Nevertheless, to what extent this transporter is involved in anthocyanin absorption needs further investigation.

Anthocyanins with different molecular size and types of sugar or acylated groups attached are absorbed intact [27,28]. As evidence, Pn 3-*O*-(2-*O*-(6-*O*-(*E*)-caffeoyl-β-D-glucopyranosyl)-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside (MW = 949) from purple-fleshed sweet potato was detected as the intact form in plasma and urine of both humans and rats [27]. However, the extent of absorption may be decreased for the complex anthocyanins [28].

Anthocyanins are absorbed and eliminated rapidly but apparent absorption efficiency is poor

After oral administration of anthocyanins from berries or berry extracts, anthocyanins were found in the blood stream within minutes. The time required to reach C_{max} ranged from 0.5 to 2 h for plasma (Table I), which is much shorter than all other sub-groups of flavonoids [11]. In contrast to other flavonoids, the proportion of anthocyanins absorbed and excreted in the urine as a percentage of the intake is quite small [4,13,29–31], perhaps much less than 0.1% of intake. Maximum plasma levels of total anthocyanins in the plasma are in the range of 1–100 nmol/l with doses of 0.7–10.9 mg/kg in human studies (Table I) [11,31–33]. The clearance of anthocyanins from the circulation is sufficiently rapid so that by 6 h, very little is detected in the plasma [33,34].

However, the apparent absorption efficiency differs depending upon whether one measures disappearance from the gastrointestinal tract or appearance in the blood or excretion in the urine. Wu et al. [35] observed that about 58% of a dose of anthocyanins disappeared from the gastrointestinal tract within 4 h after a meal as determined by recovery in the duodenum, ileum, cecum and colon. However, the extent of disappearance varied considerable depending upon the anthocyanin [35] with 98% of the Cy-3-glc disappearing but only 22% of the Cy-3-sambubioside. Talavera et al. [36] found that after administration of a high concentration of blackberry anthocyanins, intact anthocyanins were observed in plasma from the gastric vein and aorta. Cy-3-glc appeared in bile after as little as 20 min. This study seemed to demonstrate that anthocyanin glycosides were quickly and efficiently absorbed from the stomach and rapidly excreted into bile as intact and metabolized forms [36]. This rapid and apparent high absorption of anthocyanins from the stomach raises several questions as to the disposition of anthocyanins once they are absorbed. Grape anthocyanins can reach the brain within minutes of their introduction into the stomach [37]. In another paper, Mv-3-glc appeared in both portal and systemic plasma of rats after only 6 min [38]. Anthocyanins are capable of permeating the gastric mucosa, possibly through a bilitranslocase-mediated mechanism [38]. In addition to the stomach, Talavera et al. [39] found that anthocyanins were also absorbed efficiently from the small intestine. The absorption was influenced by the chemical structure of the anthocyanin and varied from 10.7

Table I. Pharmacokinetics of anthocyanins in plasma (serum) after single dose administration in human, pig and rat.

Species	Source	Anthocyanin*	Dose (μmol)	C_{max} (nM)	C_{max}/dose (nM/μmol)	T_{max}(h)	AUC/dose (nM h/μmol)	$T_{1/2}$(h)	References
Human	Eldeberry extract	Cy-3-glc	863	42.5	0.05	1.1	–	1.5	
		Cy-3-sam	492	38.9	0.08	1.2	–	2.2	[33]
	Red wine	Mv-3-glc	139	1.4	0.01	0.8	2.08	–	
	Dealcoholized red wine	Mv-3-glc	118	1.7	0.01	1.5	1.81	–	[34]
	Red grape juice	Mv-3-glc	238	2.9	0.01	2.0	2.78	–	
	Black currant concentrate	Dp-3-rut	182	73.4	0.40	1.8	1.58	3.18	
		Cy-3-rut	137	46.3	0.34	1.5	1.23	3.45	
		Dp-3-glc	69	22.7	0.33	1.5	1.00	4.19	[32]
		Cy-3-glc	24	5.0	0.21	1.3	0.38	1.34	
	Red grape juice	Cy-3-glc	7	0.9	0.14	0.5	0.19	1.61	
		Dp-3-glc	107	13.2	0.12	0.5	0.24	1.72	
		Mv-3-glc	266	99.4	0.37	0.5	0.55	1.50	
		Pn-3-glc	180	59.0	0.33	0.5	0.60	1.63	[55]
		Pt-3-glc	35	33.6	0.96	0.5	1.88	1.68	
	Red wine	Mv-3-glc	376	37.7	0.10	1.5	0.22	1.80	
		Pn-3-glc	39	27.2	0.70	1.5	1.71	1.83	
		Pt-3-glc	76	25.7	0.34	1.5	0.80	2.15	
	Black currant juice	Dp-3-rut	98	2.0	0.02	1.0	0.04	1.63	
		Cy-3-rut	75	1.5	0.02	1.0	0.03	2.0	
		Dp-3-glc	69	1.7	0.03	1.0	0.03	1.96	[96]
		Cy-3-glc	18	1.0	0.05	1.0	0.03	1.68	
	Elderberry extract	Cy-3-glc	100	11.2	0.11	1.5	0.21	1.95	
		Cy-3-sam	176	17.7	0.10	1.5	0.22	1.81	
	Chokeberry extract	Cy-3-gal	1094	23.4	0.02	2.5	0.06	$<$1.35	
		Cy-3-ara	418	8.9	0.02	3.5	0.13	$<$1.67	[60]
Human	*Hibiscus sabdariffa* L. extract	Cy-3-samb	108	3.8	0.04	1.5	0.08	–	
		Dp-3-samb	137	2.1	0.02	1.5	0.03	–	[97]
	Dry black raspberry	Cy-3-glc	301	4.8	0.02	1.1	0.05	2.5	
		Cy-3-samb	148	3.7	0.03	2.2	0.15	6.2	[98]
		Cy-3-rut	1618	37.8	0.02	1.6	0.10	2.0	
		Cy-3-xylrut	354	9.4	0.03	2.6	0.15	3.4	
Pig	Dry marion blackberry	Cy-3-glc	1452	36.3	0.04	1.0	0.07	–	[43]
		Cy-3-rut	221	15.5	0.07	1.0	0.18	–	
	Dry elderberry	Cy-3-glc	1504	49.9	0.03	1.0	0.11	–	
		Cy-3-samb	858	121.7	0.14	1.0	0.56	–	[45]
Rat	Red fruit ACN extract	Cy-3-glc	217	3490	16.1	0.25	16.6	–	[11]
	Cy-3-glc	Cy-3-glc	900 μmol/kg, BW †	310	–	0.5	–	–	[12]
	Black currant concentrate	Dp-3-rut	800 μmol/kg, BW	580	–	2.0	–	0.79	
		Cy-3-rut	800 μmol/kg, BW	850	–	0.5	–	1.36	[32]
		Cy-3-glc	800 μmol/kg, BW	840	–	0.5	–	2.08	

Table I – *continued*

Species	Source	Anthocyanin*	Dose (μmol)	C_{max} (nM)	C_{max}/dose (nM/μmol)	T_{max}(h)	AUC/dose (nM h/μmol)	$T_{1/2}$(h)	References
	Sweet potato extract	Pn-3-caf -sop-5-glc	40 μmol/kg, BW	50	–	0.5	–	0.68	[99]
	Cy-3-glc	Cy-3-glc	223 μmol/kg, BW	180	–	0.25	–	–	[63]
	Dp-3-glc	Dp-3-glc	152 μmol/kg, BW	285	–	0.4	–	1.2	[100]

* Abbreviations: Cy, cyanidin; Dp, delphinidin; Pn, peonidin; Pg, pelargonidin; Pt, petunidin; Mv, malvidin; glc, glucoside; gal, galactoside; ara, arabinoside; xyl, xylose; rut, rutinoside; sam, sambubioside; sop, sophoroside; caf, caffeoyl. † BW, body weight.

(Mv 3-glucoside) to 22.4% (Cy 3-glucoside). In a more recent paper in which the intestinal absorption of anthocyanins was studied using an *in vitro* chamber model [40], the authors found that the highest absorption of anthocyanins occurred with jejunual tissue (55.3 ± 7.6%). Minor absorption occurred with duodenal tissue (10.4 ± 7.6%), with no absorption recorded in tissue from the ileum or colon. Two peaks of Dp-3-glc at 15 and 60 min were observed in the plasma of rats after oral administration of 100 mg Dp-3-glc/kg body weight [41,42]. The second peak seemed to appear in relation to the time at which the anthocyanins reached the small intestine from the stomach, indicating that Dp-3-glc was likely absorbed from the jejunum as well as from the stomach.

Nature of chemical structure alters absorption of anthocyanins

It has consistently been observed that the glycone, sugar moiety and acylated groups impact the absorption of anthocyanins. In a study investigating absorption and metabolism of anthocyanins in weanling pigs after Marion blackberry feeding, Wu et al. [43] observed that the total urinary recovery of the four original anthocyanins plus their related metabolites was 0.087 ± 0.034% for Cy-3-glc, 0.084 ± 0.026% for Cy-3-rut, 0.583 ± 0.229% for Pg-3-glc and 0.036 ± 0.011% for an unknown acylated Cy anthocyanin, respectively. Pg-3-glc had a 8-fold higher apparent absorption rate than Cy-3-glc. This result may partly explain the extremely high total urinary excretion of Pg-3-glc and its metabolites (1.8 ± 0.29%) in human subjects after strawberry consumption [44]. In a follow-up report by Wu et al. [45], weanling pigs were given a freeze-dried powder of black currant to study the influence of aglycones and sugar moieties on the absorption and metabolism of anthocyanins. Four anthocyanins, Dp-3-glc, Cy-3-glc, Dp-3-rut and Cy-3-rut were found to be the major anthocyanins in black currant. Using total urinary excretion (parent anthocyanins plus metabolites) as an estimate of apparent absorption, Cy anthocyanins were always higher than that of Dp anthocyanins; whereas for the same aglycones, total urinary excretion of anthocyanidin rutinosides were higher than that of anthocyanidin glucosides (Dp-3-glc, 0.022 ± 0.007%; Cy-3-glc, 0.074 ± 0.011%; Dp-3-rut, 0.071 ± 0.021; Cy-3-rut, 0.106 ± 0.027%). Moreover, in the same study, total urinary excretion of Cy-3-samb and Cy-3-samb-5-glc were found to be higher than Cy monoglycosides. Mulleder et al. [46] observed a greater urinary excretion of Cy-3-samb than Cy-3-glc (0.014 vs. 0.004% of dose). More recently, two papers were published regarding the bioavailability of black raspberry in pigs [35] and human subjects [47]. In both studies, higher total urinary excretion of more complex Cy anthocyanins (Cy-3-samb, Cy-3-samb-5-glc or Cy-3-rut) compared to simple Cy-3-glc was observed. In contrast to the higher urinary excretion of Cy-3-rut compared to Cy-3-glc, the absorption of Cy-3-rut in both stomach [36] and small intestine [48] was found to be lower than that of Cy-3-glc. Our hypothesis is that the higher urinary excretion of Cy-3-rut may be a result of its increased stability rather than its increased absorption from the gut relative to Cy-3-glc. Wu et al. [43] detected an unknown acylated anthocyanin from blackberries in pig urine and demonstrated that urinary recovery of the acylated anthocyanin was lower than that of nonacylated anthocyanins. This finding was supported by a study [28] in which raw and cooking purple carrots were ingested by human subjects. Acylation of Cy (eg. Cy-3-(2″-xyl-6″-feruloyl-glc-galactoside)) in this case resulted in an 11–14-fold decrease in anthocyanin recovery in urine and an 8–10-fold decrease in anthocyanin recovery in plasma.

Anthocyanin degradation in lower GI tract

The potential role of microbial metabolism of anthocyanins in the lower gastrointestinal tract (cecum and colon) is often overlooked. Anthocyains that are not absorbed in the stomach or small intestine may be transferred to the colon. In addition, absorbed anthocyanins could also be excreted with bile into the colon. Thus, the colon might play a very important role in absorption and metabolism of anthocyanins. Wu et al. [35] found that at 4 h after pigs were given black

raspberry, 41.7 ± 4.9% of the total anthocyanins were recovered in the GI tract, mostly in the lower GI tract. Like other flavonoids [25], anthocyanins have been shown to be degraded by gut microflora to phenolic compounds [49,50]. However, the fate of individual anthocyanins in the lower GI tract is very different, depending on their structures. In a study looking at the anthocyanins in fecal or cecal contents [50], the authors observed that losses in the intestinal contents were high for anthocyanin glucosides, moderate for galactosides, and negligible for arabinosides or xylosides. Acylation or diglucosylation enhanced anthocyanin stability. Wu et al. [45] found that Cy di- or tri-glycoside exhibited a higher recovery (from 43.7 ± 6.4% of Cy-3-samb-5-glc to 78.2 ± 17.2% of Cy-3-samb), than Cy-3-glc which exhibited an extremely low recovery of only 1.7 ± 1.0% within the GI tract. This may be explained due to the more complex anthocyanins being more resistant to gut microflora and thus more stable in the environment of the lower GI tract.

A large number of flavonoids and other phenolic compounds have recently been identified in colonic water of the human [51]. Some of the phenolic or aromatic compounds were probably generated after ring fission from flavonoids by human gut microflora. Compared to polyphenolic flavonoids, the monophenolics may be present in considerable excess both in terms of concentration and number within the colon [51]. Within the gastrointestinal tract, the biological effects of monophenolics may be equal or more important than that of the polyphenolics. Anthocyanidin glycosides have been shown to be hydrolyzed by the intestinal microflora within 20 min–2 h of incubation depending on the sugar moiety [52]. Due to the high instability of the liberated anthocyanidin aglycones at neutral pH, primary phenolic degradation products were detected within 20 min of incubation. Further metabolism of the phenolic acids was accompanied by demethylation. Keppler [52] suggested that because of their higher chemical and microbial stability, phenolic acids and/or other, not yet identified, anthocyanin metabolites may be responsible for the observed antioxidant activities and other physiological effects *in vivo*. The formation of phenolic acids, as a major stable degradation product, provides an important hint as to the fate of anthocyanins *in vivo* [53]. However, due to the complexity of phenolic composition, it is hard to determine what compounds are actually generated from anthocyanins. Labeled anthocyanins are thus necessary to determine the degraded compounds which were generated from anthocyanins. Considerable work remains to be done relative to the potential absorption, metabolism and biological effects of the phenolic acids.

Other dietary components alter anthocyanin absorption

Other dietary components or the food matrix may also play a role in the absorption of anthocyanins. Alcohol was regarded as a possible factor impacting absorption of flavonoids. However, in a study in which human subjects were given both red wine and dealcoholized red wine at a similar dose, there was no difference in apparent absorption (Table I) [34]. This result was later confirmed by another study using isolated small intestine [54]. However, when a similar single oral dose of anthocyanins was given from either red wine or red grape juice to human subjects [55], the total urinary anthocyanin excretion following red grape juice administration was found to be significantly higher than that from red wine ingestion (0.23 vs. 0.18%) [55]. The total area under the curve of plasma anthocyanin concentrations also was greater for red grape juice compared to red wine (3.5 vs. 2.7 nM h/μmol) (Table I). Thus, alcohol may have little or possible even a detrimental effect on anthocyanin absorption.

When anthocyanins were given along with a high-fat diet, the maximum anthocyanin concentration in the plasma was reached 1–2 h later [56]. Ingestion of sucrose led to a delay and a reduced amount of anthocyanins in urine [46,57], whereas the addition of alcohol had no effect on the total excretion of anthocyanins. A delay was detected in the peak plasma concentration anthocyanin when anthocyanins were ingested along with a carbohydrate-rich meal (rice cake) [58]. This may indicate that either high-fat or a carbohydrate-rich diet may prolong or delay the transit of anthocyanins through the GI tract or interact at the absorption site to delay absorption, but not necessarily affect their total absorption.

Anthocyanin metabolism

Knowledge accumulated especially in the last 2–3 years has indicated that the major metabolites of anthocyanins recovered in urine were glucuronidated and/or methylated conjugates [29,35,43,45,47,59–61]. Sulfate conjugates, another common form of metabolites found with other flavonoids, were only reported as metabolites of anthocyanins in two studies thus far and they were present at extremely low concentrations [44,61]. Biotransformation enzymes involved may include UDP-glucuronosyl transferase, UDP-glc dehydrogenase or catechol-*O*-methyltransferase (COMT). These enzymes are located in the small intestine, liver or kidney. Depending on the chemical structure, anthocyanins could exist mainly as native forms or metabolites in blood and urine, whereas most other flavonoids are generally recovered as metabolites.

Glucuronidation of anthocyanins

Glucuronidated metabolites were first found by Wu et al. [29] in a study of the absorption and metabolism of anthocyanins from elderberry in elderly women.

Two glucuronidated metabolites were detected: Pn monoglucuronide and Cy-3-glc monoglucuronide. Since then, glucuronidation has been demonstrated to be a major metabolic pathway of anthocyanins. However, the extent of glucuronidation is significantly affected by aglycone, substitution pattern and amount of anthocyanins consumed. In a series of studies conducted by Wu et al. [35,43,45], glucuronidation of anthocyanins with different aglycone and glycoside substitutions were systematically studied in the weanling pig model. Cy and Pg anthocyanins were converted to glucuronidated conjugates, but no detectable glucuronidated conjugates were found for Dp anthocyanins. The amount of metabolites recovered from anthocyanidin diglycosides (eg. Cy-3-rut or Pg-3-rut) was lower than that of the original intact Cy-3-rut, whereas the amount of metabolites from Cy-3-glc and Pg-3-glc in the urine was much higher than their original forms. Pg-3-glc had a much higher apparent conversion to glucuronidated conjugates ($0.53 \pm 0.22\%$ of metabolites vs. $0.05 \pm 0.01\%$ of the parent Pg-3-glc) than Cy anthocyanins as indicated by urinary excretion. These findings were largely in accordance with other reports [59,60,62–64]. The exact mechanism whereby the glucuronide can be formed is an open question. Two pathways were proposed explaining the formation of Cy monoglucuronides (Figure 3) [45]. The first possibility is that the glucuronide is formed directly from Cy-3-glc by UDP-glc dehydrogenase; the second one requires the hydrolysis of Cy glycosides to Cy. However, evidence from the ratio of Cy-3-glc to Cy monoglucuronide in the urine supported the second possibility as the most likely, if not the only, pathway [45]. Recently, anthocyanin aglycones of Cy and Pn were detected in plasma of rats [48], which gives further support to the second possibility.

Methylation of anthocyanins

Miyazawa et al. [11] first observed the presence of methylated form of Cy-3-glc in the liver of rats following the consumption of red fruit anthocyanins, but was not able to detect conjugated or methylated anthocyanins in plasma of humans. Similar results were also obtained by Tsuda [12]. This methylated Cy-3-glc (Figure 4) was further identified as Pn-3-glc by Felgines et al. [65] and Wu et al. [29]. These results indicate that the formation of the methylated metabolites likely takes place mainly in the liver through the COMT reaction. Since the catechol structure is necessary for COMT activity, it is predictable that no methylated metabolites for Pg, Pn and Mv anthocyanins have been observed. Using the pig as a model, both 3′-*O*-methyl and 4′-*O*-methyl esters of Cy were identified in urine [43], indicating both the 3′- and 4′-hydroxyl group in the B-ring could be conjugated with a methyl group (Figure 4). However, the 3′-*O*-methyl ester, which leads to the formation of Pn, is the predominant form. Ichiyanagi [41] found methylation of Dp which occurred only at the 4′-hydroxyl group in the B-ring (Figure 5). No 3′- or 5′-methyl derivatives were found in urine or liver. As to Pt, no studies addressing the methylation of this anthocyanidin have been reported. A summary of the pathways of anthocyanin methylation and glucuronidation is presented in Figure 5.

Dose effects on anthocyanin metabolism

Due to the low apparent bioavailability, the dose of anthocyanins commonly used has generally been high. However, increasing the dose may not necessarily result in an increase in anthocyanin absorption. In a recent study [28], anthocyanin absorption in humans

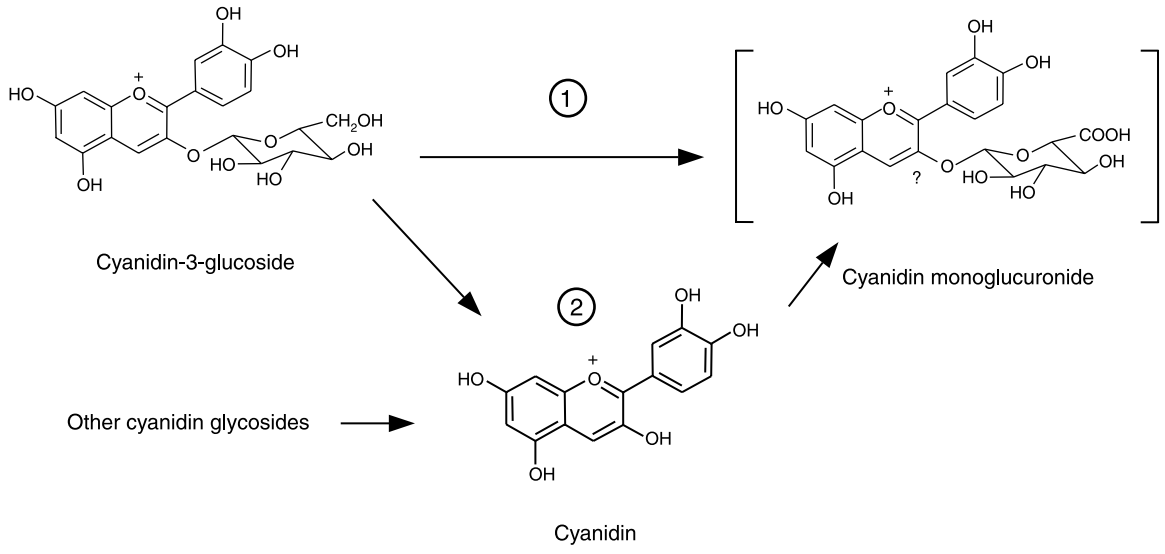


Figure 3. Two possible pathways of the formation of Cy monoglucuronide from Cy-3-glc or other Cy glycosides.

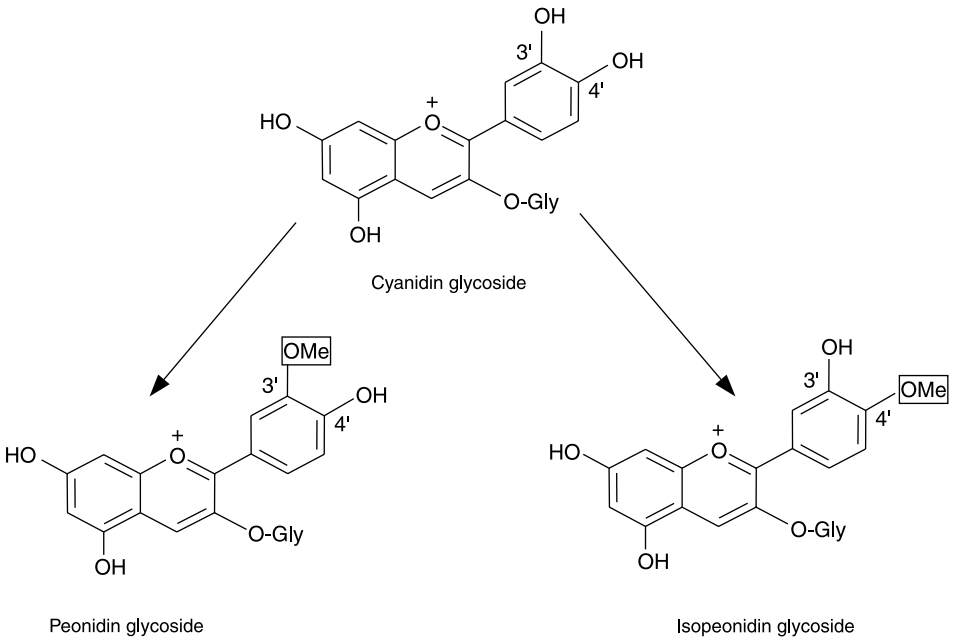


Figure 4. Formation of two methylated conjugates from Cy-3-glc. 3′-methylation of Cy leads to the formation of Pn; methylation at the 4′-positon of Cy leads to the formation of isoPn.

was maximal at a dose of 350 μmol (or possibly less) of both acylated and nonacylated anthocyanins, suggesting saturation of the absorption mechanism. In other studies, the amount of Cy-3-glc consumed appeared to have an effect on the metabolism of Cy-3-glc via methylation and/or glucuronidation [45]. With a relatively high dose (>130 μmol/kg BW), an equal or greater amount of methylation occurred relative to glucuronidation, whereas at much lower doses (5–10 μmol/kg BW) a greater proportion of the

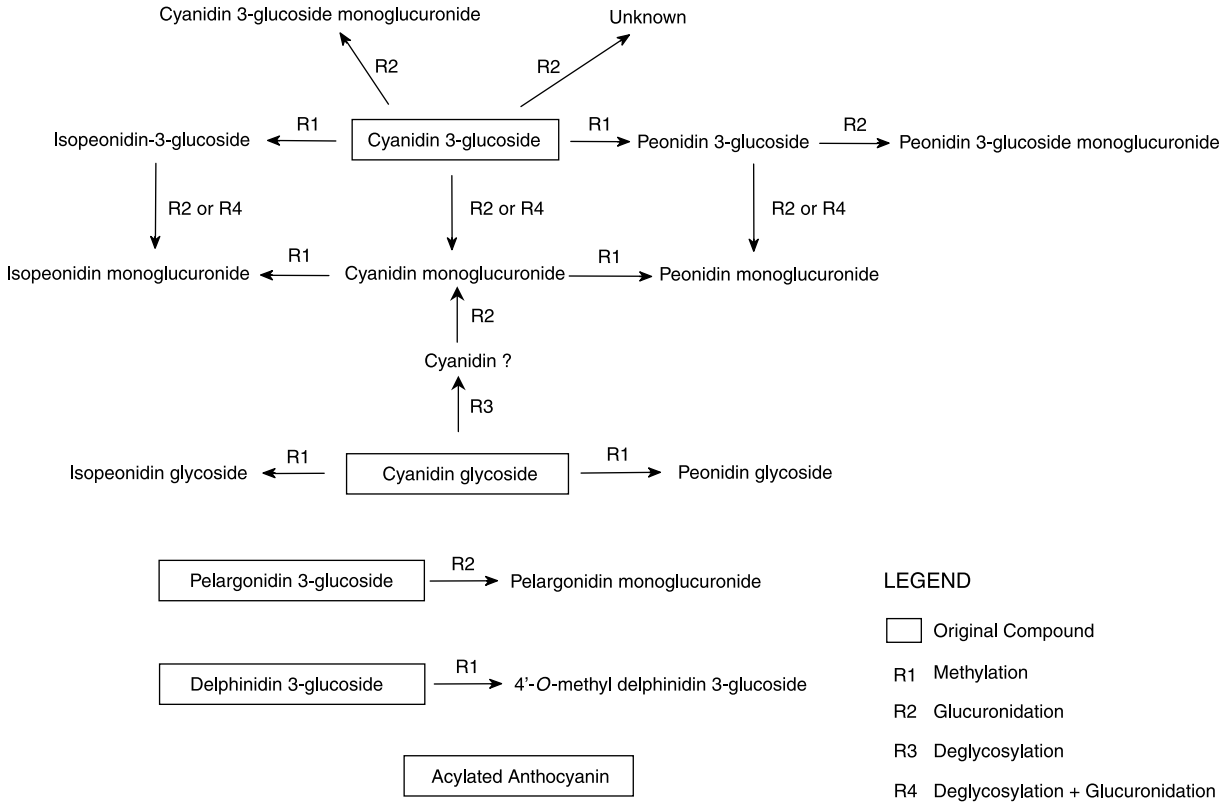


Figure 5. Proposed metabolic pathways for anthocyanins.

Cy-3-glc was conjugated with glucuronide relative to being methylated [45].

Species differences in anthocyanin metabolism

A large number of the studies completed to date on the metabolism and conjugation of flavonoids have been done using the rat as a model. However, it is becoming apparent that for some of the flavonoids, the rat may not be the best model for extrapolation to human metabolism [66]. Protocatechuic acid (PCA), which may be produced by degradation of Cy, was present in the plasma of the rat at concentrations 8-fold higher than that of Cy-3-glc [12]. We have not detected PCA in the plasma of humans following anthocyanin consumption [29,33], nor has it been reported by others in any other publications in humans. More recently, a similar urinary anthocyanin profile after elderberry consumption from pig and human was observed [45], which may indicate that the pig may be a good model for the study of the bioavailability of anthocyanins.

Tissue distribution of anthocyanins

If anthocyanins are responsible for health effects, tissue distribution of the parent anthocyanin or a metabolite becomes important in understanding the mechanism(s) of the effect. Since the early observations of the effects of blueberry on cognitive performance [67], some recent publications have investigated the question as to whether anthocyanins reach the brain. Talavera et al. [48] found native blackberry anthocyanins and Pn 3-glc concentrations that reached 0.25 ± 0.05 nmol/g of tissue in the brain. Tissues of the stomach contained only native blackberry anthocyanins (Cy-3-glc and Cy-3-pentoside), while in other organs such as the jejunum, liver and kidney, native and methylated anthocyanins as well as conjugated anthocyanidins (Cy and Pn monoglucuronides) were identified. The liver contained the highest proportion of methylated forms.

Andres-Lacueva et al. [68] identified several anthocyanins (Cy-3-gal, Cy-3-glc, Cy-3-arab, Mv-3-gal, Mv-3-glc, Mv-3-arab, Pn-3-arab and Dp-3-gal) in various regions of the brain including the cerebellum, cortex, hippocampus and striatum of aged rats fed a 2% blueberry diet for ten weeks. Correlation analyses revealed a relationship between Morris water maze performance (a measure of spatial learning and memory) in blueberry fed rats and the total number of anthocyanin compounds found in the cortex. These findings suggest that these compounds may deliver their antioxidant and signal modifying capabilities centrally.

Passamonti et al. [37] found intact anthocyanins, extracted from grape (*Vitis vinifera*), in the brain at concentrations of 192 ng/g after 10 min residence of anthocyanins within the stomach at a dose of 8 mg/kg body weight. These results demonstrate a rapid movement of grape anthocyanins from the stomach to the mammalian brain.

Recently, Mohsen et al. [69] demonstrated that Pg or its metabolites were present in kidney, liver, brain and lung tissues 2 h post-gavage, but spleen and heart did not contain detectable levels of Pg or its metabolites. Pg glucuronide was the major metabolite in kidney and liver (0.5 and 0.15 nmol Pg equivalents/g tissue, respectively) with smaller amounts present in the lung. Compared to other anthocyanins, Pg is metabolized to a much greater extent to the glucuronide form [43,69] and was present in the urine only as the glucuronide [69].

Anthocyanins and brain function

A mulberry fruit extract containing Cy-3-glucoside has been shown *in vivo* to have neuroprotective effects using a mouse-brain-injury model with transient middle cerebral artery occlusion. *In vitro* a 1% HCl–MeOH mulberry fruit extract was shown to have a cytoprotective effects on PC12 cells that had been exposed to hydrogen peroxide and the extract also inhibited the cerebral ischemic damage caused by oxygen glc deprivation in PC12 cells [70].

Ramirez [71] demonstrated in rats fed lyophilised berries (blueberry or bilberry) (3.2 mg/kg per day of anthocyanins) that there was significantly enhanced short-term memory, but not long-term memory in an inhibitory avoidance task, and induced an increase in the number of crossings in the first exposure to the open field. However, treated rats did not present any improvement of memory retention in open field habituation. The feeding of these berries to rats also improved working memory in the radial maze, with significant differences observed during sessions 1–2 and 4, but diet did not alter reference memory in this task.

Other studies have looked at the effects of dietary blueberry and other antioxidant sources on brain function. However, the results have not been linked specifically to anthocyanins. In one study, age-related changes in temporal speed in the primary auditory cortex appeared to reversed by dietary supplementation with blueberry phytochemicals [72]. A short-term (10 weeks) dietary blueberry intervention resulted in improved HSP70-mediated protection against a number of neurodegenerative processes in the brain [73]. The blueberry diet completely restored the HSP70 response to LPS in old rats at 90 and 240 min following LPS treatment [73]. In earlier studies, dietary blueberry supplementation reversed the deleterious effects of aging on motor behavior and neuronal signaling in senescent rodents [74]. Blueberry-fed (from 4 months of age) APP + PS1 transgenic mice showed no deficits in Y-maze

performance (at 12 months of age) with no alterations in amyloid beta burden, indicating that it may be possible to overcome genetic predispositions to Alzheimer disease through diet [75]

Although no clinical trials have been completed to date, results suggest that these berries may be beneficial in the prevention of memory deficits, one of the symptoms related to Alzheimers disease, and corroborate previous findings showing that the flavonoids present effect several learning paradigms.

Anti-obesity effects of anthocyanins

Tsuda and coworkers [76] published data on the effects of anthocyanins from purple corn (PC) in the prevention of obesity and the amelioration of insulin resistance is a mouse model. Mice were fed a control diet, a diet rich in Cy-3-glc from PC, a high fat (HF) diet or HF + PC diet for 12 weeks. Dietary PC anthocyanins significantly suppressed the HF diet-induced increase in body weight gain, and white and brown adipose tissue weights. The HF diet induced hyperglycemia, hyperinsulinemia and hyperleptinemia which were normalized in rats fed HF + PC. An increase in the tumor necrosis factor (TNF-α) mRNA level occurred in the HF group and was normalized by dietary PC. These results suggest that dietary PC may ameliorate HF diet-induced insulin resistance in mice. PC suppressed the mRNA levels of enzymes involved in fatty acid and triacylglycerol synthesis and lowered the sterol regulatory element binding protein-1 mRNA level in white adipose tissue. These down-regulations may contribute to the decreased triacylglycerol accumulation in white adipose tissue.

Further work by Tsuda and coworkers [77] evaluated the gene expression profile in isolated rat adipocytes treated with anthocyanins (100 nM Cy-3-glc, or Cy) for 24 h *in vitro*. A total of 633 genes or 427 genes were up-regulated (>1.5-fold) by the treatment of adipocytes with Cy-3-glc or Cy, respectively. The up-regulated genes included lipid metabolism and signal transduction-related genes, however, the altered genes were somewhat different between the Cy-3-glc and Cy treated groups. Based on the gene expression profile, up-regulation of hormone sensitive lipase and enhancement of the lipolytic activity were demonstrated by the treatment of adipocytes with Cy-3-glc or Cy. Although this data may have identified new responsive genes with potentially important functions in adipocytes related to obesity and diabetes, additional investigation is needed. *In vivo*, adipocytes are not likely to be exposed to the aglycone because of the instability of the aglycone.

Jayaprakasam and coworkers [78], also using *in vitro* methods, looked at several anthocyanins (Cy-3-glc, Dp-3-glc, Cy-3-gal and Pg-3-gal) and anthocyanidins (Cy, Dp, Pg, Mv and Pt) for their ability to stimulate insulin secretion from rodent pancreatic beta-cells (INS-1 832/13) in the presence of 4 and 10 mM glc. Pg-3-gal, and its aglycone, Pg, caused a 1.4-fold increase in insulin secretion at 4 mM glc concentration. The rest of the anthocyanins and anthocyanidins tested had only marginal effects on insulin at 4 and 10 mM glc concentrations [78]. However, further studies *in vivo* provide additional evidence that anthocyanins may ameliorate obesity and insulin resistance in mice fed a high-fat diet [78]. Cy-3-gal and Pg-3-gal are the predominate anthocyanins from Cornelian cherries (*Cornus mas*) which were used in these studies. Mice (C57BL/6) initially fed a high-fat diet for 4 weeks and then switched to a high-fat diet containing anthocyanins (1 g/kg of high-fat diet) for an additional 8 weeks were compared to mice fed only the high-fat diet. The high-fat diet induced glc intolerance was prevented by dietary anthocyanins. The anthocyanin-treated mice also showed a 24% decrease in weight gain and decreased lipid accumulation in the liver, including a significant decrease in liver triacylglycerol concentration. Anthocyanin treated mice had elevated insulin levels and pancreatic islet architecture and insulin staining were preserved. Overall, these data suggest that anthocyanins purified from Cornelian cherries have biological activities that improve certain metabolic parameters associated with diets high in saturated fats and obesity.

Antioxidant effects of anthocyanins

Intake of anthocyanins (34.5 mg/day), contained in chokeberry juice, was found to lower blood parameters of redox status in rowers performing a physical exercise during a 1-month training camp. After the supplementation period, the concentrations of TBARS in blood samples collected 1 min after the exercise test and following a 24-h recovery period were significantly lower in the subjects receiving chokeberry juice than in the control group. The investigators suggested that the anthocyanins may have enhanced the endogenous antioxidant defense system [79].

Indomethacin-induced gastric mucosal damage in the rat was accompanied by the development of oxidative stress as evidenced by the accumulation of malondialdehyde. Pretreatment of rats with *Aronia melanocarpa* fruit juice (5, 10 and 20 ml/kg), which is rich in anthocyanins, decreased the gastric lesions caused by indomethacin [80].

In a double-blind, placebo-controlled, crossover study [81], the effects of blackcurrant anthocyanin intake on peripheral circulation during rest and during typing work was investigated. The results of this study suggest that intake of blackcurrant anthocyanins may improve shoulder stiffness caused by typing work by increasing peripheral blood flow and reducing muscle fatigue.

Biological responses of cells in culture to anthocyanins

In vitro responses of cells in culture to anthocyanins or anthocyanidins are summarized in Table II. Emphasis has been placed on data appearing during the past year. Earlier reviews can be consulted for some of the earlier literature [6,82,83]. The functional areas where anthocyanins have some effects include cardiovascular function, cancer prevention and anti-inflammatory effects. Results from *in vitro* studies must be interpreted in light of the high concentrations of anthocyanins that are usually used *in vitro* and because of the instability of anthocyanins at physiological pH. As is indicated in Table I, the C_{max} in plasma for anthocyanins following a meal is in the range of 1 nM– $<$ 1 μM but the concentrations used *in vitro* are usually in the 5–200 μM range. The studies of Bell and coworkers [84] (Table II) are some of the first to demonstrate effects of anthocyanins at levels that are reasonably achieved in plasma. Furthermore, few investigators have followed anthocyanin concentrations in the cell culture systems. Most anthocyanins will have a half-life in culture of less than 5 h (Wu and Prior, unpublished data). Any observed effects in extended incubations of anthocyanins may or may not be a direct result of the anthocyanins, but may result from degradation products.

Some studies [85–87] have used the aglycone of the anthocyanin. The aglycone form is generally not found *in vivo* in the plasma or urine nor in the tissues that have been studied. Thus, interpretation and extrapolation of results from studies of this nature using relatively high concentrations and the aglycone form of anthocyanins need to be interpreted with these factors in mind. Of five anthocyanidins studied, Dp and Cy were shown to inhibit LPS-induced COX-2 expression, but Pg, Pn and Mv did not. Furthermore, Dp suppressed the activation of mitogen-activated protein kinase (MAPK) including c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 kinase. LPS induced COX-2 expression by activating MAPK pathways and Dp suppressed COX-2 by blocking MAPK-mediated pathways with the attendant activation of nuclear factor-κB (NF-κB), activator protein-1 (AP-1) and C/EBPdelta. These findings suggest that only anthocyanidins with the *ortho*-dihydroxyphenyl structure may have anti-inflammatory properties through the inhibition of MAPK-mediated COX-2 expression [87]. A crude extract of *Aronia* (chokeberry) suppressed LPS-induced iNOS and COX-2 protein expressions in RAW 264.7 cells *in vitro* in a dose-dependent manner which lead to the suppression of the production of NO, PGE_2 and TNF-α [88].

In hepatoma cells (HepG(2)), Dp, Cy and Mv exhibited strong growth inhibitory effects. Dp, furthermore, induced apoptotic cell death accompanied by up-regulation of Bax and down-regulation of Bcl-2 protein. Dephinidin-induced apoptotic cell death and DNA fragmentation, which was blocked by N-acetyl-l-cysteine and catalase, suggesting that the death signaling was triggered by oxidative stress. These results suggest that induction of apoptosis by anthocyanidins might be a pivotal mechanism in their cancer chemoprevention action [86]. Increased apoptosis and/or decreased cell proliferation in the presence of anthocyanins (~10–200 μM range) is a common observation in various cancer cell lines [86,89–94] (Table II).

In cultured human umbilical vein endothelial cells (HUVECs), the aglycones of Cy and Dp both increased the protein level of eNOS, but Dp showed the major effect raising eNOS protein in a dose-dependent manner, which produces nitric oxide, a vascular tone regulator. An anti-proliferative effect induced by Dp was detected in HUVECs. Dp also showed a higher antioxidant activity than Cy. In this model, as well, it seemed that the greater biological activity of Dp, compared with Cy, was due to the presence of the three-hydroxyl groups on the B-ring in the molecular structure of Dp [85].

Increased efflux of cholesterol from mouse peritoneal macrophages treated with 1–100 μM anthocyanins (either Cy-3-glc or Pn-3-glc) was observed, which was mediated, at least in part, by the activation of peroxisome proliferator-activated receptor gamma-liver X receptor alpha-ABCA1 signaling pathway [95].

Although much is being learned from the *in vitro* work with anthocyanins, additional research is needed to confirm that the observed effects are in deed due to the anthocyanins.

Conclusion

Progress and interest in anthocyanins and their biological effects has increased immensely during the past decade. Ten years ago there were three papers published dealing with anthocyanins in an animal or human model. This past year there were over 60 manuscripts published. We have learned much about their absorption and metabolism, but there are still major gaps in our knowledge. From the studies to date, it seems that the absorption process for anthocyanins can be saturated and that acylation of anthocyanins lowers their apparent absorption. Diglycosides in the form of sambubioside or rutinoside on the anthocyanidin seems to impart increased stability to the anthocyanin molecule. However, until we have radiolabeled anthocyanins available, it is going to be difficult to fully understand what happens to 60–90% of the anthocyanins that disappear from the gastrointestinal tract. Degradation definitely accounts for a part of this disappearance but the exact amount we do not know. The fact that it is different for the various aglycones and may be modified further by the nature

Table II. Biological response observed *in vitro* with anthocyanins, anthocyanidins or extracts high in anthocyanins.

Health effect	Anthocyanin source	Concentration	Cells	Citation
Cardiovascular				
1. ↑ Endothelial dependent relaxation	*Aronia* (chokeberry) or bilberry	0.05 mg/l	Porcine coronary arteries	[84]
2. ↓ HUVEC proliferation (angiogenesis)	Del-3-(cr)-5-glc	5–200 μM	Human umbilical vein endothelial cells (HUVEC)	[101]
3. ↑ Cholesterol efflux and ABCA1 mRNA expression	Cy-3-glc, Peon-3-glc	1–100 μM	Mouse peritoneal macrophages	[95]
4. ↓ Proliferation, ↑ eNOS	Delphinidin	50–100 μM	Human umbilical vein endothelial cells (HUVEC)	[85]
Cancer				
1. ↑ Apoptotic cell death	*Hibiscus sabdariffa*	0–4 mg/ml	HL-60	[89]
2. ↑ Apoptosis and cytodifferentiation	Cy-3-glc	NA	HL-60 and Jurkat	[90]
3. ↓ *In vitro* invasiveness of cancer cells	Mulberry; Cy-3-glc; Cy-3-rut	NA	A549 human lung carcinoma cells	[91]
4. ↑ Apoptosis, ↓ ERK activity, ↑ p38 kinase expression	Mal-3-glc	0–200 μM	Human gastric adenocarcinoma cells	[92]
5. ↑ Apoptosis, ↑ c-Jun N-terminal kinase cascade	Delphinidin	10–200 μM	Human hepatoma cell line-HepG(2)	[86]
6. ↓ Cell proliferation	Blueberry extract	50–100 μg/ml	HT-29 and CaCo-2 colon cancer cells	[93]
7. ↓ Tumor cell proliferation with anthocyanidins, no effect with anthocyanins	4 anthocyanins, 5 anthocyanidins	12.5–200 μg/ml	AGC, HCT-116, MCF-7, NCI H460 & SF268	[94]
Oxidative damage				
1. ↓ DNA fragmentation due to mycotoxin	Cy-3-glc	50 μM	Hep G2 (Hepatoma) CaCO-2	[102]
2. ↓ Free radical production and genomic DNA damage	Cy-3-glc	125 & 250 μM	Human fibroblast	[103]
3. ↓ UVA-induced reactive oxygen species	Cy-3-glc	NA	Human keratinocyte cell line (HaCaT)	[104]
Anti-Inflammatory				
1. ↓ COX-2 expression	Delphinidin	25–100 μM	Macrophage cell line RAW 264.7	[87]
1. ↓ NO, PGE2, COX-2	*Aronia* (chokeberry)	1–100 mg *Aronia* extract	Macrophage cell line RAW 264.7	[88]

of the glycosylation of the aglycone further complicates our understanding of this process. Much more information is becoming available indicating that anthocyanins may play an important role in health promotion in terms of obesity, cardiovascular health, anti-inflammatory and anti-cancer effects, however, much remains to be done with *in vivo* studies in animal models and human clinical trials.

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