A Mixed Fruit and Vegetable Concentrate Increases Plasma Antioxidant Vitamins and Folate and Lowers Plasma Homocysteine in Men[1,2]

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ABSTRACT Fruit and vegetable consumption is inversely associated with coronary heart disease (CHD) risk. The aim of the present study was to determine the effect of supplementation with dehydrated juice concentrates from mixed fruit and vegetables on selected plasma vitamins and antioxidant status. We assessed CHD risk by measuring the concentrations of homocysteine, lipids, lipoproteins, glucose and insulin. Men were recruited to participate in a randomized double-blind, crossover trial with 2 periods of 6 wk, separated by a 3-wk wash-out period. Supplementation with the encapsulated mixed extract (Juice Plus) was compared with physically similar placebo capsules. Thirty-two men (13 smokers, 19 nonsmokers) completed the study with a mean compliance of 88%. Compared with placebo, supplementation increased the concentrations of plasma β-carotene (0.24 $\pm$ 0.15 vs. 1.12 $\pm$ 0.70 μmol/L; mean $\pm$ SD; $P < 0.0001$), retinol (1.87 $\pm$ 0.33 vs. 2.00 $\pm$ 0.43 μmol/L; $P < 0.05$), α-tocopherol (16.8 $\pm$ 7.3 vs. 19.3 $\pm$ 6.8 μmol/L; $P < 0.01$), ascorbic acid (72.1 $\pm$ 19.4 vs. 84.1 $\pm$ 13.5 μmol/L; $P < 0.002$) and folic acid (24.5 $\pm$ 10.0 vs. 44.9 $\pm$ 16.9 nmol/L; $P < 0.0001$). Plasma homocysteine was reduced (8.2 $\pm$ 1.5 vs. 7.6 $\pm$ 1.1; $P < 0.05$) and inversely related ($r = -0.40$, $P < 0.001$) with serum folate concentrations. Plasma vitamin C was positively correlated with the resistance of LDL to oxidation ($r = 0.26$, $P < 0.05$) and the plasma ferric reducing/antioxidant power (FRAP) tended to be greater after supplementation than after the placebo period (1125.5 $\pm$ 144.1 vs. 1180.3 $\pm$ 158.1 μmol/L; $P < 0.065$). Plasma glucose, insulin and lipid concentrations were unaffected. Responses of smokers and nonsmokers did not differ. In the absence of dietary modification, supplementation with a fruit and vegetable concentrate produced responses consistent with a reduction in CHD risk. J. Nutr. 133: 2188–2193, 2003.

KEY WORDS: • *fruit and vegetable concentrates* • *vitamins* • *homocysteine*

Cardiovascular disease is the largest single cause of death in the United States, the UK and Australia (1–3). It is a multifactorial disease, but a fundamental environmental factor is diet (4). Recent large-scale cohort studies have shown that the risk of coronary heart disease (CHD)[4] is inversely associated with the intake of vegetables in men (5) and a higher intake of fruit and vegetables is associated with lower risk of myocardial infarction in women (6). This inverse association between fruit and vegetable intake and CHD appears to be consistent across different geographical locations and in populations that differ in lifestyle, gender and age (7–9). These observations have led to continued support for the recommendation to increase the consumption of fruit and vegetables (10–12).

There remains considerable scientific debate about the contribution of specific dietary constituents to the reduction in CHD risk. It has been suggested that the association of a lower rate of CHD with the intake of fruit and vegetables is attributed to the displacement of foods that are high in Na, energy density and fat, particularly saturated fat (11,13). In addition, fruit and vegetables are recognized sources of a number of nutrients that may interact to reduce LDL cholesterol (soluble fiber and plant sterols) (14,15), blood pressure (lower Na/K ratio) (16) and homocysteine (folate) (17), and to improve antioxidant status (18) and endothelial function (19).

The aims of the present study were to determine the bioavailability of selected carotenoids and vitamins from a mixed concentrate of dehydrated fruit and vegetable juice and the effects on antioxidant status, homocysteine, plasma lipids and insulin resistance in men. We tested the hypothesis that increasing fruit and vegetable intake, in the form of a mixed concentrate derived from juice, has the potential to lower CHD risk in the absence of dietary displacement.

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[4] AA, ascorbic acid; CHD, coronary heart disease; FFQ, food-freqency questionnaire; FRAP, ferric reducing antioxidant power; HOMA, homeostasis model assessment.

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SUBJECTS AND METHODS

Study design. Subjects participated in a double-blind, randomized, placebo-controlled, crossover trial, consisting of two intervention periods of 6 wk separated by a 3-wk wash-out period. They were randomly assigned to one of two groups to receive first either a mixed fruit and vegetable supplement produced from dehydrated juice concentrates (4 capsules; Juice Plus, Natural Alternatives International, San Marcos, CA) or a placebo. The supplement was provided as two capsules of "fruit blend" in the morning and two capsules of "vegetable blend" in the evening (composition shown in **Table 1**). The placebo capsules were physically identical to the intervention capsules but contained alfalfa and beetroot to mimic the colors of the vegetable and fruit supplements, respectively. At the beginning of each period, subjects were provided with a known number of capsules and asked to return any that were unused. These were counted to determine apparent compliance. Subjects were asked to maintain their habitual dietary and exercise patterns and to refrain from commencing any form of vitamin or herbal supplementation during the intervention.

Blood samples were obtained before supplementation (d −1, 1), on d 21 and at the end of the intervention period (d 41, 43). After the 3-wk wash-out period, the protocol for blood collection was repeated. In all, each subject provided 10 blood samples over a period of 15 wk. During wk 1 of the trial, the subjects' heights and weights were measured and their dietary intakes were determined by questionnaire.

The study design was approved by the University of Sydney Human Ethics Review Committee (00/05/26) and all subjects gave written informed consent before their participation.

Subjects. Men were recruited between November 2000 and May 2001 by advertisements in local area newspapers and notice boards on the University campus. All potential subjects were interviewed by using a short questionnaire that sought information about their general health, family history, use of medication or nutritional supplement and smoking status. The subjects had to meet the following criteria: age 18–50 y; not using nutritional supplements or medication; BMI < 30 kg/m^2; no reported chronic disease; and no history of metabolic disease. We recruited smokers and nonsmokers.

Blood sampling. On each occasion, blood samples from subjects were collected into vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) as follows: EDTA-coated tubes for the subsequent analysis of plasma lipids and homocysteine; heparin-coated tubes for the analysis of ascorbic acid (AA), carotenoids and antioxidant activity; and untreated tubes for the analysis of serum folate. All samples were taken between 0730 and 1100 h from an antecubital vein. Subjects were fasting (10–12 h) and in the supine position during blood collection. Blood samples were kept on ice and centrifuged within 1 h at 1500 × *g* for 8 min at 4°C. Plasma or serum was removed and placed on ice. Samples for the analysis of AA were stabilized immediately by the addition of metaphosphoric acid (250 g/L) then stored at −80°C. Aliquots of plasma and serum were protected from light and stored at −80°C until subsequent analysis.

TABLE 1

Micronutrient composition of the fruit and vegetable supplements[1]

Nutrient	Fruit blend[2]	Vegetable blend[3]
	unit/capsule	
Folic acid, *μg*	50	150
Vitamin C, *mg*	75	25
Vitamin E, *mg*	13.2	13.2
Carotenoids, *mg* β-carotene equivalents	3	3
Bioflavonoids, *mg*	50	50

[1] Composition provided by Natural Alternatives International, San Marcos, CA.

[2] Blend of apple, orange, pineapple, pawpaw, cranberry, acerola cherry and peach juice extracts.

[3] Blend of carrot, barley, parsley, beetroot, broccoli, cabbage, spinach, tomato juice extract and kale leaf powder.

Biochemical analyses. Before analysis, all frozen samples were thawed and centrifuged at 1000 × *g* for 10 min at 4°C, to sediment any precipitated fibrin. Samples were analyzed in batches at the end of the study.

Plasma β-carotene, lycopene, lutein + zeaxanthin, retinol and α-tocopherol were measured in a single assay by HPLC (Hewlett Packard 1050 series, Sydney, NSW, Australia) with multiwave detection (Programmable Electrochemical Detector 1049A, Hewlett Packard) (20). Plasma AA was measured by HPLC with UV detection (21).

The antioxidant activity of plasma was determined by the ferric reducing/antioxidant power (FRAP) assay (22) and analyzed in an automated system (Cobas Fara, Roche Diagnostic Systems, Sydney, Australia) (23).

Serum folate concentrations were determined by the electrochemiluminescence method (Elecsys Folate Reagent Kit, Roche Diagnostics). Plasma homocysteine was measured by the fluorescence polarization immunoassay method (IMX system, Abbott Laboratories, North Ryde, NSW, Australia).

Plasma cholesterol and triacylglycerol concentrations were assayed using enzymatic colorimetric assay kits (CHOD-PAP, Boehringer Mannheim, Sydney, Australia; Triacylglycerol PAP Unimate 5, Roche Products); HDL cholesterol concentrations were assayed following selective precipitation of apolipoprotein B–containing lipoproteins (24). LDL cholesterol was estimated using the SI version of the Friedewald equation (25).

To determine the propensity of LDL to oxidative modification, LDL were isolated from plasma using a single spin ultracentrifugation at 450,000 × *g* for 3.5 h at 4°C in a Beckman 70.1 Ti rotor (Beckman Instruments, Palo Alto, CA) (26). LDL were eluted through two sequential desalting columns (Econo-Pac 10DG, Bio-Rad Laboratories, Hercules, CA). The protein concentration of the LDL preparation was determined using a modified Lowry method (27). LDL (200 μg LDL protein) were incubated with $CuSO_4$ (final concentration 5 μmol/L) for 5 h at 37°C in a UV-visible spectrophotometer (Cary 300, Varian Australia, Melbourne, Australia). Absorbance at 234 nm was recorded automatically at 1-min intervals and oxidation was determined as the change in absorbance (28). The lag phase, which represents the resistance to oxidation, and the maximum change in absorbance were determined. Absorbance values were converted into diene concentrations by using the extinction coefficient for conjugated dienes at 234 nm [29,500 $(mol/L)^{-1}cm^{-1}$].

Plasma glucose concentrations were analyzed enzymatically (Glucose HK Unimate 5, Roche Products). Plasma insulin concentrations were measured with a solid-phase RIA kit using antibody-coated tubes and internal controls (Coat-a-Count insulin, Diagnostic Products, Los Angeles, CA). The homeostasis model assessment for insulin resistance (HOMA-IR) (29) was calculated as follows: fasting insulin (mU/L) × fasting glucose (mmol/L)/22.5.

Dietary data analysis. All subjects completed a self-administered, semiquantitative food-frequency questionnaire [FFQ, 30] modified for the Australian diet. FFQ data were checked for outliers and data entry errors. Two subjects were excluded from the dietary assessment because of incomplete questionnaires. Dietary intakes were analyzed using a database that incorporated the Australian Tables of Food Composition (NUTTAB;31) and the USDA-National Cancer Institute Carotenoid Food Composition database (32).

Statistical analysis. The data were analyzed using the standard method for an AB/BA crossover trial (33). The differences between the beginning (mean of d −1, 1) and end (mean of d 41, 43) of each period were used to obtain summaries of changes during the Placebo and Treatment periods. Comparisons between treatments were carried out using ANOVA of a general linear model with SPSS version 10 (Chicago, IL). Differences between smokers and nonsmokers were also tested by including "smoker" as a factor in the ANOVA model. Values are expressed as means ± SD. Differences with $P < 0.05$, without adjustment for multiple comparisons, were considered significant.

TABLE 2

Baseline characteristics and dietary intake of subjects[1,2]

	All subjects	Nonsmokers	Smokers
n	32	19	13
Age, *y*	28 ± 7	26 ± 6	31 ± 9
Weight, *kg*	73.5 ± 10.4	73 ± 11.7	74 ± 8.6
BMI, *kg/m²*	23.2 ± 2.6	23 ± 2.7	24 ± 2.6
Cigarettes, *n/d*	—	—	9 ± 7.6
Energy, *kJ/d*	9099 ± 2606	8312 ± 2510	10281 ± 3232
Protein, *%en*	17.5 ± 3.8	16.8 ± 2.9	18.5 ± 4.7
Fat, *%en*	30.8 ± 6.8	30.6 ± 7.5	31.0 ± 5.3
Carbohydrate, *%en*	46.9 ± 8.1	48.3 ± 9.1	44.7 ± 5.3
Alcohol, *%en*	5.6 ± 4.3	5.1 ± 4.6	6.4 ± 3.5
Vitamin C, *mg*	140.8 ± 84.9	130.8 ± 90.0	155.8 ± 78.1
Folic acid, *μg*	347.7 ± 123.6	326.2 ± 115.4	379.9 ± 133.4
Retinol equivalents, *μg*	1056 ± 456	956 ± 428	1206 ± 472
β-Carotene, *μg*	3964 ± 2555	3736 ± 2463	4307 ± 2761
Lutein + Zeaxanthin, *μg*	708.3 ± 362.8	617.8 ± 320.9	844.0 ± 392.7
Lycopene, *μg*	4803 ± 3511	4560 ± 3550	5166 ± 3576

[1] Values are mean ± SD.
[2] %en, % of energy.

RESULTS

Thirty-eight men were selected for the study. Six subjects withdrew during the first phase of supplementation: four cited logistic reasons and were unable to meet the study schedule, two withdrew because they had reconsidered their participation. Thirty-two subjects completed the trial and their baseline data are presented in **Table 2**. Smokers and nonsmokers did not differ in nutrient intakes (Table 2) or any of the biochemical variables. It is noteworthy that the intakes of vitamin C and folate were beyond the recommended dietary intake [40 mg and 200 μg, respectively; (34)] due mainly to the consumption of fruit juice and foods fortified with folic acid.

The degree of compliance was estimated as the difference between the number of capsules issued and the number of capsules returned. Apparent compliance was 87.4 ± 10.2 and 88.7 ± 10.0% for the placebo and supplementation periods, respectively. There were no differences in compliance of smokers and nonsmokers.

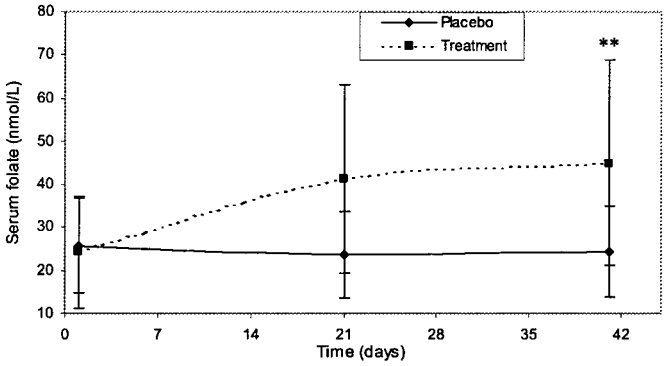

FIGURE 1 Serum folate response in men before and after supplementation with a placebo and a mixed fruit and vegetable concentrate (Treatment) in a crossover trial. Values are means ± SD, $n = 32$. **Means at a time differ, $P < 0.0001$.

Consistent with the high degree of compliance, plasma AA concentrations increased significantly after supplementation ($P < 0.002$; **Table 3**). Similarly, plasma β-carotene, retinol and α-tocopherol concentrations increased significantly (Table 3). The change in α-tocopherol, but not retinol, remained significant after adjustment for cholesterol concentrations. Plasma lycopene and lutein + zeaxanthin concentrations were not affected by supplementation (Table 3).

Despite the high intake of folic acid at baseline, serum folate concentrations increased by 78.8% after supplementation ($P < 0.0001$; **Fig. 1**) and were correlated with plasma β-carotene ($r = 0.49$, $P < 0.0001$) and α-tocopherol ($r = 0.26$, $P < 0.05$). Concurrent with the rise in serum folate, plasma homocysteine decreased by 8.4% ($P < 0.05$; **Fig. 2**); the reduction occurred in 23 of 32 subjects with a maximum change of −3.1 μmol/L. Plasma homocysteine concentrations were correlated negatively with serum folate ($r = -0.40$, $P < 0.001$).

Plasma glucose (5.05 ± 0.64 vs. 5.08 ± 0.58 mmol/L), insulin (53.9 ± 20.0 vs. 51.0 ± 15.8 pmol/L) and insulin resistance, as measured by the HOMA index, were unaffected by supplementation. Similarly, plasma total cholesterol, total

TABLE 3

Concentrations of plasma carotenoids, antioxidant vitamins and FRAP in men before and after supplementation with a placebo and a mixed fruit and vegetable concentrate (treatment) in a crossover trial[1,2]

	Beginning	Placebo End	Change	Beginning	Treatment End	Change
			μmol/L			
Ascorbic acid	68.2 ± 18.0	72.1 ± 19.4	3.8 ± 20.3	63.5 ± 21.6	84.1 ± 13.5	20.6 ± 18.2[a]
α-Tocopherol	18.51 ± 7.2	16.82 ± 7.25	−1.69 ± 4.45	17.69 ± 6.35	19.29 ± 6.81	1.6 ± 5.30[b]
Retinol	1.97 ± 0.43	1.87 ± 0.33	−0.10 ± 0.32	1.94 ± 0.37	2.00 ± 0.43	0.09 ± 0.25[c]
β-Carotene	0.40 ± 0.38	0.24 ± 0.15	−0.16 ± 0.28	0.22 ± 0.12	1.12 ± 0.70	0.91 ± 0.64[d]
Lycopene	0.66 ± 0.28	0.59 ± 0.26	−0.06 ± 0.30	0.68 ± 0.26	0.61 ± 0.22	−0.07 ± 0.19
Lutein + zeaxanthin	0.31 ± 0.13	0.27 ± 0.14	−0.30 ± 0.06	0.30 ± 0.13	0.28 ± 0.14	−0.01 ± 0.08
FRAP	1145.0 ± 168.9	1125.5 ± 144.1	−19.5 ± 105.9	1144.5 ± 149.3	1180.3 ± 158.1	36.2 ± 121.5

[1] Values are mean ± SD, $n = 32$.
[2] FRAP, ferric reducing ability of plasma.
[a] Different from placebo, $P < 0.002$.
[b] Different from placebo, $P < 0.01$.
[c] Different from placebo, $P < 0.05$.
[d] Different from placebo, $P < 0.001$.

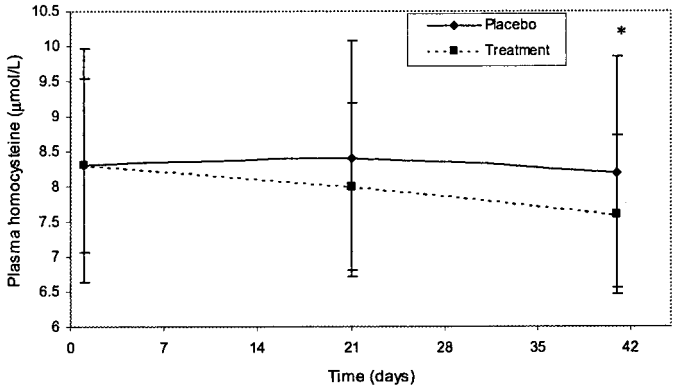

FIGURE 2 Serum homocysteine response in men before and after supplementation with a placebo and a mixed fruit and vegetable concentrate (Treatment) in a crossover trial. Values are means ± SD, $n = 32$. *Means at a time differ, $P < 0.05$.

triglyceride, LDL cholesterol and HDL cholesterol were not affected. The resistance of LDL to oxidative modification as assessed by the lag phase (54.5 ± 14.1 and 57.0 ± 11.1 min, end of placebo and supplementation periods, respectively) and conjugated dienes (38.9 ± 9.3 and 39.2 ± 8.2 μmol/L, respectively) were not affected although the antioxidant capacity of plasma as assessed by FRAP, tended to be greater after supplementation than after the placebo period ($P < 0.065$; Table 3). The lag phase was significantly correlated with plasma AA concentration ($r = 0.26$, $P < 0.05$).

DISCUSSION

The results of the present study show that a mixed fruit and vegetable supplement produced from dehydrated juice provides a source of bioavailable vitamins and β-carotene, and lowers the risk of heart disease by decreasing the concentration of plasma homocysteine. The latter represents a reduction in CVD risk that occurred in the absence of dietary displacement.

Plasma AA reaches a plateau at ~80 μmol/L, possibly due to a threshold for its urinary excretion (35). In the present study, the fruit and vegetable concentrate provided 200 mg vitamin C/d and resulted in plasma AA of 84 μmol/L, a mean increase of 20 μmol/L. This magnitude of change is consistent with previous trials that utilized vitamin C in purified form (36), as part of fruit juice (37) or fruit and vegetable concentrate (38,39). The increase in plasma AA contributes to the antioxidant capacity of plasma as demonstrated by a decrease in malondialdehyde (38) and lipid peroxides (40), or a slight increase in the plasma FRAP in the present study. Although differences in plasma antioxidants have been shown previously in smokers compared with nonsmokers (41), it is possible that the self-reported average cigarette smoking in the present study was not high enough to result in differences between the two groups.

The oxidizability of LDL ex vivo has been shown to be inhibited by AA in some (42,43) but not all (36,44) intervention studies. Those studies utilized large doses of vitamin C and the discrepancy between the results is not explained by differences in the plasma AA concentrations. Hence, the weak positive association between plasma AA and the lag phase of LDL oxidation demonstrated in the present study is unexpected. One possible explanation is that vitamin C is acting as a surrogate biomarker of other constituents of the mixed fruit and vegetable concentrate (45). It has been shown recently that the antioxidant activity of fruit and vegetables can be explained in part by vitamin C and in part by other dietary components, such as phytochemicals (46).

Large interindividual differences in the plasma response to β-carotene are due to the presence of hypo- and hyperresponders (47) and large variations in bioavailability associated with the dietary source (48,49). The latter is due to numerous factors but is thought to be due primarily to the entrapment of β-carotene within subcellular organelles such as chloroplasts (48). Intervention studies in which β-carotene intake was increased through fruit juice (37) or fruit and vegetables (49,50) showed small changes in plasma β-carotene. In the present study, plasma β-carotene increased by a mean of 0.9 μmol/L, a fourfold increase from baseline. The high degree of bioavailability is consistent with previous trials that utilized purified supplements (49,51) or preparations similar to that used in the present study (38,40,52). The enhanced bioavailability may be explained by the dose provided in the capsules (Table 1), their consumption with meals that contained fat (48) and by the disruption of cellular structures that occurs in the commercial processing of the fruit and vegetable concentrate (J.A. Wise, Natural Alternatives International, San Marcos, CA, personal communication).

There is limited information on the absorption of folate from whole foods (53,54); however, disruption of the cellular matrix may be one of the factors that enhances its bioavailability. The concentration of plasma folate rose to 20.5 nmol/L after supplementation with 400 μg of folic acid derived from the supplement (Table 1). The change in plasma folate is similar to that seen when folate intake is increased in the form of food (560 μg) or as food + supplement (460 μg) (55). Although disruption of the matrix improves bioavailability from some foods (49), on balance, the bioavailability of folate from the mixed fruit and vegetable concentrate that was used in the present study appears to be similar to that expected from a diet that contains a range of vegetables and citrus fruits (55). The increase in folate intake was associated with a significant reduction in plasma homocysteine, with a mean change of −8.4%. Similar associations have been reported in other studies in which folate was consumed as a supplement (56,57), in fortified foods (57) or as an increase in the intake of fruit and vegetables (55,57).

The majority of prospective studies have shown that dietary or plasma folate levels are associated with a reduced risk of CHD in individuals who are free from the disease at baseline (58). The benefits of folic acid are explained largely by its homocysteine-lowering effect, which produces favorable outcomes in endothelial function as assessed by flow-mediated vasodilation or hemostatic markers (19). The maximal reduction in homocysteine occurs with supplements of 200 μg of folic acid (56) or a total intake of ~400 μg (17). The present results show that plasma homocysteine concentrations decreased when folate intake was increased in the form of a fruit and vegetable supplement (400 μg), and this occurred despite the high dietary intake of folic acid at baseline (347 μg).

There are numerous factors that influence the homocysteine response. These include defects in the enzymes involved in homocysteine metabolism or suboptimal intake of micronutrients such as vitamins B-6 and B-12 (59). Although tobacco use is associated with hyperhomocysteinemia (59), there were no significant differences in the homocysteine response between smokers and nonsmokers in the present study.

Plasma concentrations of lipids, glucose and insulin and the HOMA index were not affected by supplementation with the fruit and vegetable concentrate. Similarly, it has been shown

that a vegetable/fruit concentrate (39), purified monoterpene supplements (60) and selected phytochemicals with cytochrome P_{450}– inducing activity (61) do not affect plasma lipids. From this limited number of observations, we suggest that minor constituents of fruit and vegetables at the doses provided are unlikely to be involved in the regulation of cholesterol or glucose homeostasis. These observations support the view that fruit and vegetables decrease plasma lipid concentrations by displacing cholesterol-raising constituents of the diet, particularly saturated fat (11,13).

In conclusion, supplementation with a mixed fruit and vegetable concentrate increases plasma AA, β-carotene and folate, and lowers plasma homocysteine. A large body of evidence suggests that fruit and vegetables lower the risk of heart disease, and the results of the present study suggest that one of the mechanisms, in the absence of dietary displacement, is the reduction in plasma homocysteine.

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