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Effect of Vegetarianism and Smoking on Vitamin B_{12}, Thiocyanate, and Folate Levels in the Blood of Normal Subjects

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Summary

Vitamin B_{12}, thiocyanate, and folate levels in the blood were estimated in 69 apparently normal subjects, of whom 26 were non-vegetarian non-smokers, 19 non-vegetarian smokers, 15 vegetarian non-smokers, and nine vegetarian smokers. The serum total (cyanide-extracted) B_{12} level (value A) ranged from 105 to 728 pg/ml, with a mean of 292 pg/ml. The highest values were found in non-vegetarian non-smokers and the lowest in vegetarian smokers. There was no significant difference in value A between smokers as a group and non-smokers as a group. On the other hand, in vegetarians value A was very significantly lower than in non-vegetarians regardless of their smoking habits.

It is suggested that A may represent both the protein-bound and free forms of vitamin B_{12} in the blood, and B mainly the free B_{12}, which may be the physiologically active form. The plasma thiocyanate level varied from 1·0 to 15 μmol/100 ml, being, as expected, much higher in smokers (mean 8·20 μmol/100 ml) than in non-smokers (mean 2·02 μmol/100 ml). There was a rough correlation between falling B_{12} levels and rising thiocyanate levels. The serum folate level ranged from 2·75 to 15·75 ng/ml, and was slightly but significantly higher in vegetarians (mean 6·60 ng/ml) than in non-vegetarians (mean 4·79 ng/ml), reflecting the greater content of folate in a vegetarian diet.

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Introduction

It was suggested by Wokes and Picard in 1955 and by Wokes in 1958 that vitamin B_{12} contributed to the detoxication of exogenous cyanide, such as that derived from tobacco smoke. Cyanide probably participates in normal metabolic processes (Boxer and Rickards, 1952) and possibly combines with B_{12} to form cyanocobalamin. Heaton *et al.* (1958) reported reduced serum B_{12} levels in pipe smokers with visual impairment and suggested that this was related to the pathogenesis of tobacco amblyopia.

It was recognized by Spray (1955) and by Boger *et al.* (1955) that unless cyanide was added to the buffer used to extract serum B_{12} in the *Lactobacillus leichmannii* assay, the results obtained were spuriously low. Matthews (1961, 1962) clearly showed that this was owing to loss of the vitamin in the protein precipitate. Subsequently, even in their radioisotopic assay, Matthews *et al.* (1967) advocated the use of acetate-cyanide buffer for the extraction of the total cobalamins from the serum.

The estimation of microquantities of cyanide and thiocyanate in the blood was first carried out by Aldridge (1945). Using this method, Wilson and Matthews (1966) found higher plasma thiocyanate values in smokers than in non-smokers, both groups being otherwise normal. They further showed that the proportion of non-CN-extracted serum B_{12} was higher in smokers than in non-smokers. They suspected a significant relation between plasma thiocyanate concentration and the level of non-CN-extracted B_{12}.

The interrelationships between smoking and serum B_{12} levels might be expected to be affected by B_{12} intake, which is generally lower in vegetarians than in meat-eaters. Hence the objectives of the investigations reported here were: (1) to observe the serum B_{12}, plasma thiocyanate, and serum folate levels in four groups of apparently healthy subjects—non-vegetarian non-smokers, non-vegetarian smokers, vegetarian non-smokers, and vegetarian smokers; and (2) to study the interrelationships between these three compounds, and especially the relation of smoking to the levels of total (CN-extracted) and non-CN-extracted cobalamins.

Subjects and Methods

Of the 69 subjects studied, 39 were drawn from apparently healthy laboratory workers and doctors; the remaining 30 were drawn from patients' relatives, also apparently healthy, who had come to donate blood at the blood bank. None of them had any evidence of anaemia. These 69 subjects fell into the following four groups: (1) 26 non-vegetarian non-smokers, (2) 19 non-vegetarian smokers, (3) 15 vegetarian non-smokers, and (4) 9 vegetarian smokers. All smokers smoked at least 10 cigarettes a day; there were no pipe smokers. All the vegetarians took milk and dairy products and may thus be regarded as lacto-vegetarians. None of these subjects took any vitamin supplements.

Serum B_{12} levels were estimated by the isotopic assay described by Matthews *et al.* (1967). Essentially, the procedure consisted in extraction of total cobalamins from the subject's serum with acetate-cyanide buffer at pH 4·5, this being subsequently called "total (CN-extracted) B_{12}" or value A. Another part of the serum was treated identically but with acetate buffer alone, this being subsequently called "non-CN-extracted B_{12}" or value B. Both these extracts were assayed after the addition of ^{57}Co-B_{12} as the label, normal human serum as the binding protein, and albumin-coated charcoal to separate the bound and free fractions, which were counted in a well-type gamma-ray spectrometer. The free:bound ratio was calculated for both the sample and the standard solutions of B_{12} and plotted against concentrations of the standards; the value in the sample was read off from this graph.

Plasma thiocyanate levels were estimated spectrophotometrically by the method of Aldridge (1945) and serum folate levels by a modification of the *L. casei* assay procedure of Baker and Frank (1968).

Results

SERUM VITAMIN B_{12}

As shown in Table I there was a stepwise downward gradation of mean levels of total (CN-extracted) serum B_{12} (value A) from the non-vegetarian non-smokers to the non-vegetarian smokers, vegetarian non-smokers, and vegetarian smokers, though the differences were mostly insignificant. There were, however, highly significant differences ($P < 0·01$) between vegetarian and non-vegetarian non-smokers and between vegetarian and non-vegetarian smokers. It was obvious that the nature of the diet was responsible for the differences between the four main groups and not the smoking habit. This was clearly confirmed when the mean value of A in smokers, regardless of diet, was not found to be significantly different from that in non-smokers, whereas the level in vegetarians, regardless of smoking habit, was found to be highly significantly lower than that in non-vegetarians (Table II).

The mean value of A for all subjects taken together was 292 pg/ml (range 105–728 pg/ml) (Table II). When the patients' relatives were considered separately from the laboratory workers and doctors, however, the former showed a significantly lower mean value than the latter (Table II, third column); the ranges were 105–365 pg/ml and 154–728 pg/ml respectively.

There was no significant difference between smokers and non-smokers or between vegetarians and non-vegetarians in respect of non-CN-extracted serum B_{12} (value B). There were, however, significant differences both between smokers and non-smokers and between vegetarians and non-vegetarians when B was expressed as a percentage of A ($B/A \times 100$) (Table II). Vegetarianism as well as smoking appeared to bring about a rise of $B/A \times 100$. This is discussed below.

TABLE I—*Serum Vitamin B_{12}, Thiocyanate, and Folate Levels in Four Groups of Normal Subjects (Mean ± S.D.). Numbers of Subjects are given in Parentheses*

Groups	Serum Total (CN-extracted) B_{12} (pg/ml) (A)		Serum Non-CN-extracted B_{12} (pg/ml) (B)	B/A × 100	Plasma Thiocyanate (μmol/100 ml)	Serum Folate (ng/ml)
Non-vegetarian non-smokers	365 ± 200 (26)	415 (15) 297 (11)	141 ± 80 (25)	39 ± 5·7	1·75 ± 0·83 (25)	5·01 ± 2·27 (20)
Non-vegetarian smokers	314 ± 161 (19)	365 (10) 263 (9)	150 ± 60 (14)	48 ± 10·1	8·86 ± 4·43 (15)	4·50 ± 2·18 (13)
Vegetarian non-smokers	204 ± 45 (15)	213 (9) 190 (6)	103 ± 38 (14)	51 ± 14·3	2·47 ± 0·77 (15)	6·45 ± 4·02 (12)
Vegetarian smokers	173 ± 68 (9)	210 (5) 127 (4)	104 ± 39 (8)	60 ± 5·3	6·98 ± 3·24 (8)	6·85 ± 1·37 (7)
Non-vegetarian non-smokers vs Non-vegetarian smokers	*					†
Vegetarian non-smokers vs Vegetarian smokers	†			†	§	†
Non-vegetarian non-smokers vs Vegetarian non-smokers	‡					
Non-vegetarian smokers vs Vegetarian smokers						
Non-vegetarian non-smokers vs Vegetarian smokers					§	
Non-vegetarian smokers vs Vegetarian non-smokers						

* $P < 0·01$.
† N.S.
‡ $P < 0·05$.
§ $P < 0·001$.
Paired figures in column 3 indicate the two different types of subject (see text).

TABLE II—*Comparison between Smokers and Non-smokers and between Vegetarians and Non-vegetarians (Mean ± S.D.). Numbers of Subjects are given in Parentheses*

Groups	Serum Total (CN-extracted) B_{12} (pg/ml) (A)		Serum Non-CN-extracted B_{12} (pg/ml) (B)	B/A × 100	Plasma Thiocyanate (μmol/100 ml)	Serum Folate (ng/ml)
Non-smokers	306 ± 179 (41)	339 (24) 259 (17)	128 ± 70 (39)	42 ± 10·7	2·02 ± 0·95 (40)	5·29 ± 3·07 (32)
Smokers	270 ± 152 (28)	313 (15) 221 (13)	133 ± 58 (22)	49 ± 10·9	8·20 ± 4·10 (23)	5·55 ± 2·23 (20)
Non-vegetarians	345 ± 185 (45)	395 (25) 280 (20)	145 ± 73 (39)	42 ± 7·9	4·41 ± 4·40 (40)	4·79 ± 2·21 (33)
Vegetarians	192 ± 57 (24)	212 (14) 165 (10)	103 ± 38 (22)	54 ± 12·9	3·00 ± 2·93 (23)	6·60 ± 3·24 (19)
All subjects	292 ± 169 (69)	329 ± 187 (39) 243 ± 129 (30)	130 ± 65 (61)	45 ± 11·5	4·28 ± 3·93 (63)	5·45 ± 2·74 (52)
Non-smokers vs Smokers	*			‡		
Non-vegetarians vs Vegetarians	†			†		‡
Non-smokers vs Non-vegetarians						
Smokers vs Vegetarians						
Non-smokers vs Vegetarians						
Smokers vs Non-vegetarians						
All subjects		*				
All subjects		‡				

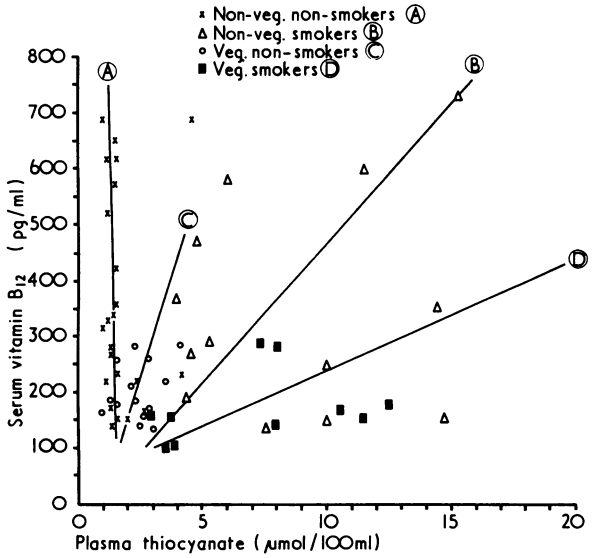
* N.S.
† $P < 0·01$.
‡ $P < 0·05$.
Paired figures in column 3 indicate the two different types of subject (see text).

For all subjects B/A × 100 ranged from 24 to 72%, with a mean of 45%. This compares with the range of 28-78% with a mean of 51% reported by Matthews (1961) using *L. leichmannii* assay. The highest mean value of B/A × 100 was among vegetarian smokers (60%) and the lowest among non-vegetarian non-smokers (39%) (Table I). Moreover, the two vegetarian smokers with the lowest total B_{12} values (105 pg/ml each) also showed raised values of B/A × 100 (60 and 62% respectively). On the other hand, in a non-vegetarian smoker with a very high plasma thiocyanate level (see below) B was only 37% of A.

PLASMA THIOCYANATE

As expected, plasma thiocyanate values were highly significantly raised in smokers when compared with non-smokers (Table II), and in vegetarian and non-vegetarian smokers separately (Table I). There was no significant difference between vegetarians and non-vegetarians (Table II). The lowest individual value was 1·0 μmol/100 ml and the highest 15·4 μmol/100 ml (in a young man of 20 who had been smoking "charas," a preparation of *Cannabis indica*, in his cigarettes).

The actual serum total B_{12} and plasma thiocyanate values in



Correlation between plasma thiocyanate and serum total vitamin B_{12}

all the 69 subjects are shown in the Chart. It is seen that non-vegetarian non-smokers, vegetarian non-smokers, non-vegetarian smokers, and vegetarian smokers, form interdigitating groups, with a trend *in that order* towards a fall in serum B_{12} and a rise in plasma thiocyanate levels.

SERUM FOLATE

Smokers and non-smokers did not differ significantly in respect of their serum folate levels, but the mean level was slightly but significantly higher in vegetarians than in non-vegetarians (Table II). The lowest individual value was 2·75 ng/ml and the highest 15·75 ng/ml; both subjects happened to be vegetarians.

Discussion

This appears to be the first time that vitamin B_{12}, thiocyanate, and folate levels in the blood have been estimated in separate groups of vegetarian and non-vegetarian smokers and non-smokers. Serum B_{12} levels in the normal population have been estimated by microbiological assay by several groups of workers from India and the West (Mollin and Ross, 1952; Dixit *et al.*, 1956; Herbert *et al.*, 1960; Satoskar *et al.*, 1961; Gupta, 1965; Baker and Frank, 1968; Jathar *et al.*, 1970). However, only Satoskar *et al.*, Gupta, and Jathar *et al.* gave comparative values for vegetarians and non-vegetarians, the former being the lower, and they did not study the relation of B_{12} levels to smoking.

The significantly lower serum total B_{12} values found by us in vegetarians than in non-vegetarians are probably a reflection of the lower intake of the vitamin on their part, meat products being rich in B_{12}. Vegetarians who can eat adequate amounts of milk and nuts, which are also rich in B_{12}, can maintain adequate serum levels. People from the lower socioeconomic groups, such as those of the apparently normal subjects who were drawn from among the relatives of the generally poor patients in our free hospital, would be expected to have a deficient intake of all these relatively expensive articles of diet and therefore to have lower serum B_{12} levels. This is precisely what was seen in this group, among whom non-vegetarians as well as vegetarians had significantly lower B_{12} values than their counterparts among the more prosperous laboratory workers and doctors (Table II).

In 11 vegans Smith (1962) found serum B_{12} values ranging from 48 to 190 pg/ml and suggested that they had remained free of symptoms of any central nervous system disorder because the major portion of the B_{12} in their serum was in the hydroxo form. He further made a comparison between veganism and tobacco amblyopia and invoked endogenous and exogenous factors interfering with cyanide metabolism in both these conditions. In a vegan who did not receive any B_{12} supplement Linnell *et al.* (1969) found a serum B_{12} value of 100 pg/ml, measured by the isotopic assay that we also used. This is comparable to the value of 105 pg/ml in two of our vegetarian smokers.

Smith (1961a, 1961b) stated that in patients with tobacco amblyopia and in heavy pipe smokers there was no significant difference between the serum B_{12} values obtained with and without added cyanide, whereas the difference was highly significant in non-smokers. He suggested that this difference represents the amount of hydroxobalamin in the blood. The effect of smoking on the serum B_{12} level is a phenomenon not amenable to any facile evaluation. Thus in our subjects the small and statistically insignificant reduction in value A in the smokers as compared with the non-smokers has to be weighed against the virtual absence of any difference in value B between these two subgroups (Table II). Hence it would appear that the statistically significant rise of B/A × 100 among smokers is a reflection of the reduction in value A as compared with non-smokers. Wilson and Matthews (1966) reported similar findings.

It is of interest that we also found the value of B in relation to A to be significantly higher in vegetarians than in non-vegetarians irrespective of smoking habit. Hence smoking was not the sole factor producing a rise of B/A × 100. To find an explanation of this the possible nature of values A and B must be discussed.

The conversion of the various forms of vitamin B_{12} to that form used as the standard—cyanocobalamin—is an essential step in the isotopic assay in order to avoid any *differential* binding of the true forms of B_{12} to the binding agent. This may also hold good for microbiological assays if the responses of the microbe to the various forms of the vitamin differ. CN-extracted B_{12} (value A) and non-CN-extracted B_{12} (value B), as determined by us or by Wilson and Matthews (1966), obviously do not represent any true forms of vitamin B_{12} in the blood. As shown originally by Lindstrand and Stahlberg (1963) and Stahlberg (1964) in normal subjects and by Linnell *et al.* (1969) in both normal subjects and patients with pernicious anaemia, uni-directional chromatography coupled with bioautography shows two constantly present forms of B_{12}—namely, methylcobalamin and a combination of hydroxocobalamin with deoxyadenosyl-cobalamin. Recently the latter two have been separated by two-directional chromatography (Linnell *et al.*, 1970).

Cyanocobalamin certainly does not represent the total

cobalamins and may be present in very small amounts or virtually absent. Lindstrand *et al.* (1966) could detect cyanocobalamin in only a minority of smokers and, when detected, it was present in relatively small amounts. Hence the conclusion is inescapable that the total (CN-extracted) B_{12} in the blood is not cyanocobalamin, as believed at one time (Wokes and Picard, 1955), but perhaps a combination of all the true forms of B_{12} mentioned above. The concentration of true cyanocobalamin in the blood may be raised in tobacco amblyopia and hereditary optic neuropathies in the presence of a normal total B_{12} level; this rise is thought to be functionally unimportant (Wilson *et al.*, 1971; Matthews 1971). The other important feature shown by bioautography is that methylcobalamin is biologically the most important fraction of vitamin B_{12} and that this is the fraction which is depleted in pernicious anaemia and not hydroxocobalamin, which may in some cases be the predominant component (Linnell *et al.*, 1969).

In view of this, the earlier suggestion of Smith (1961a, 1961b) that the difference between values A and B represents the amount of hydroxocobalamin in the blood and that this is the physiologically active fraction concerned with the detoxication of cyanide appears untenable. What values A and B actually represent in terms of the different forms of cobalamins now discovered is still unclear. It seems plausible that value A, as obtained in the present investigation, represents all the protein-bound B_{12} together with any present in the free form. It is now known that there are at least two B_{12}-binding proteins in the blood, transcobalamin I, an α-globulin described by Pitney *et al.* (1954), and transcobalamin II, a β-globulin described by Hall and Finkler (1963). Hall and Finkler (1965) later suggested than transcobalamin I might be concerned with the transport of pre-existing endogenous B_{12}, while the main function of transcobalamin II might be the transport of B_{12} shortly after its intake into the body. It is also not clear what value B represents in terms of B_{12} bound to transcobalamin I and transcobalamin II or to any other plasma protein. It appears possible that value B, as assayed with *L. leichmannii* or radioisotopically, represents free B_{12}.

Our finding that smoking is certainly not the sole factor, and perhaps not the most important factor, in producing a rise of B in relation to A suggests the possibility that B and $B/A \times 100$ may not be mere reflections of increased levels of cyanide or thiocyanate in the blood. The fact that we found $B/A \times 100$ to be maintained at a high level of about 60% even when the total value (A) had fallen as low as 105 pg/ml (in two vegetarian smokers) could suggest that B represents a physiologically active moiety which the body tries to maintain until a critical level of the total value is reached. This suggestion gains support from our recent (unpublished) finding in three patients with B_{12} neuromyelopathy of abnormally low values of A as well as of $B/A \times 100$ ($<20\%$). In two of them, on whom successive estimations could be carried out in the course of B_{12} therapy, there was first a gradual rise of $B/A \times 100$ to abnormally high levels (90%), followed a week after the last injection by a return to the normal level (50%).

Nothing need be said about the serum folate levels observed except that the significantly higher value in vegetarians than in non-vegetarians (Table II) is probably a reflection of the higher intake of folates in a vegetarian diet.

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