Neutral Steroid Concentrations in the Faeces of North American White and South African Black Populations at Different Risks for Cancer of the Colon


SUMMARY

To test the hypothesis that a high risk for cancer of the colon might be associated with high concentrations of neutral steroids in the colon and with breakdown of these compounds by intestinal bacteria, the faecal neutral steroid concentrations of 84 rural South African Black adults (a population at low risk for colonic cancer), and of 98 North American White adults (a population at high risk for colonic cancer) were compared. Not only were the concentrations of animal steroids higher in the faeces of the North Americans, but the chemical state of their faecal steroids was different from that of the Blacks. A high proportion of plant and animal steroids in the faeces of the Blacks was esterified to long-chain fatty acids, whereas in the faeces of the North Americans, most of the neutral steroids were free (non-esterified). There was considerable variation in the extent of cholesterol metabolism by the intestinal bacteria in the North Americans. This was not the case with the South African population, which was much more homogeneous with respect to cholesterol metabolism.


Diet appears to be the main environmental factor associated with a risk for cancer of the colon. The highest incidence of colonic cancer occurs among populations whose diets have a high content of meat and animal fats. The amount of crude fibre in the diet may also be involved, since a correlation between high dietary fibre content and low risk has been noted. The mechanism by which diet influences carcinogenesis is not known, but it has been suggested that dietary components might produce alterations in the chemical environment of the colon by affecting the composition and metabolic activity of the intestinal flora.

Initial studies comparing the neutral and acid steroids in the faeces of populations at different risk for cancer of the colon indicated that much greater concentrations of neutral steroids and bile acids were excreted by high-risk populations than by those of low risk, and that these compounds were more extensively metabolized by the colonic flora of the high-risk populations. These results led Hill et al. to others to postulate that a high risk for cancer of the colon might be associated with the breakdown of cholesterol and the bile acids by the intestinal flora, possibly leading to the production of carcinogenic intermediates.

The studies on which this hypothesis was based involved relatively small numbers of subjects and no details were given concerning variations in steroid metabolism within the populations studied. In a recent survey of the faecal neutral steroids of North Americans, a population known to be at high risk for cancer of the colon, two distinct patterns of conversion of cholesterol to coprostanol and coprostanone were found by one of us (T.D.W.). In the faeces of most of the subjects tested, at least half of the available cholesterol had been converted to coprostanol and coprostanone by intestinal microorganisms (high conversion pattern). In the faeces of approximately one-fourth of the subjects tested, however, little or no cholesterol had been converted (low conversion pattern). Individual conversion patterns remained constant from specimen to specimen. The existence of two patterns of bacterial metabolism of neutral steroids in the colon raised the question, in the light of the hypothesis mentioned, of whether the two groups might be at different risks for cancer of the colon.

In this article, the results of a further test of the hypothesis that neutral steroid conversion might be associated with colonic cancer are reported. The neutral steroid concentrations and cholesterol conversion patterns in the faeces of rural South African Blacks from three tribal groups were determined. Rural South African Blacks are known to be at very low risk for colonic cancer. Data from this population were compared with neutral steroid concentrations and cholesterol conversion patterns in the faeces of a group of North American Whites larger in number than the one previously reported. The extents to which cholesterol and coprostanol are esterified to long-chain fatty acids in the faeces of these two populations have also been compared. Since esterification decreases the solubility of the neutral steroids, it may affect their adsorption to dietary fibre in the colon. In addition, esterification can influence the ability of bacteria to metabolize these compounds. The extent to which neutral steroids are
esterified in the faeces of populations at different risk for colonic cancer has not previously been investigated.

SUBJECTS AND METHODS

Selection of Subjects

The rural South African Black population consisted of 30 men and 54 women who ranged in age from 20 to 43 years. One group (38 subjects) lived in Tlaseng, a village near Rustenburg, western Transvaal; the second group (23 subjects) lived in the White River area of the eastern Transvaal Lowveld; the third group (23 subjects) lived in the Highveld district of Pietersburg in the northern Transvaal. The diet of the Tlaseng group, described elsewhere, was based on cereal foods, legumes, vegetables, some milk and dairy produce and meat once or twice a week. This diet, as consumed by adults, is adequate in kilojoules and total protein, but low in fat (especially animal fat) and high in crude fibre. The diet of the other two groups was similar in composition to that of the Tlaseng group.

The North American population consisted of 63 male and 45 female subjects who ranged in age from 5 to 25 years. All members of this population lived in Blacksburg, a university town with a population of 20 000, located in the south-western part of Virginia. They ate a typical North American high meat/high fat diet, with meat once or twice a day.

Faecal Specimens

Faecal specimens were collected as previously described. Since previous studies demonstrated that neutral steroid levels and cholesterol conversion patterns remain relatively stable from specimen to specimen, only one specimen was collected from each individual. Specimens from the rural South African population were shipped to Virginia Polytechnic Institute in liquid nitrogen.

Neutral Steroid Analysis

Details of the procedure used for extraction and quantitation of neutral steroids have been given in an earlier publication. For the extraction of the neutral steroids from freeze-dried faeces we followed the method of Hill and Aries. Gas-liquid chromatography of the ‘underivatized’ neutral steroids was carried out by the method of Eneroth et al. on a Hewlett Packard 5830A gas chromatograph equipped with a flame ionization detector and an automatic injector. The column was 2 mm (ID) by 2 m and was packed with 3% QF-1 on Chromosorb Q 100/120 mesh (Applied Science Lab. Inc., State College, Pa). Alpha-cholestanol (Applied Science Lab, Inc.) was used as an internal standard. Efficiency of recovery of neutral steroids was determined for each specimen by adding (4-14C) cholesterol (New England Nuclear, Boston, Mass.) to the dry faeces before extraction, and these results were used to correct quantitation. Mean recovery of cholesterol was 91 ± 4%. Identification of major peaks was confirmed by mass spectrometry with the use of a Varian MAT model 112 median resolution instrument. Good separation of all plant and animal steroids was obtained, except for stigmasterol which could not be separated from ethylcoprostanol.

To determine what proportion of the neutral steroids was esterified use was made of a method based on that of Hirsch and Ahrens: 0.3 g of dry faeces was extracted with 10 ml of chloroform methanol (2:1, v/v). One portion which consisted of 2.5 ml (A) was evaporated to dryness, saponified and the concentration of total neutral steroids determined by the procedure described above. A second portion which consisted of 5.0 ml of the chloroform-methanol extract was evaporated to dryness, re-suspended in 3.0 ml of hexane and layered on a 7 x 70-mm column of silicic acid, Sil-B-200 (Sigma Chemical Co., St Louis, Mo.). The column was washed with 10 ml of hexane; this fraction was discarded. Fatty acid esters of the neutral steroids (B) were eluted with 20 ml of hexane ether (99:1, v/v) and the free neutral steroids (C) were eluted with 10 ml of ether. Both fractions were evaporated to dryness, saponified and the concentrations of the neutral steroids determined. To test efficiency of separation and recovery, either (4-14C) cholesterol or (4-14C) cholesteryl oleate (New England Nuclear, Boston, Mass.) was added to dry faeces and the counts per minute (CPM) in fractions B and C were determined and compared with the total CPM added to the faeces. In both cases, at least 95% of the activity was recovered in its proper fraction. The sum of neutral steroid concentrations from B and C was at least 94% of the concentration of neutral steroids determined by direct saponification (A). Concentrations of free and esterified neutral steroids in the faeces of 25 North American and 28 South African subjects were determined with this method.

Fatty acids esterified to neutral steroids were determined as follows. Fraction C was evaporated, then saponified and extracted with hexane, as described above, to remove neutral steroids. The aqueous layer which remained after neutral steroid extraction was acidified with HCl to pH 2, fatty acids were extracted with hexane and the hexane extract was evaporated to dryness. Methyl esters were made by adding 1 ml of 14% BF3-methanol (Applied Science Lab. Inc., State College, Pa) and heating at 100°C for 2 min. Methyl esters were extracted into hexane-containing methyltricosanate (Applied Science Lab. Inc.) as an internal standard. (Carboxyl-14C) tripalmitin (New England Nuclear, Boston, Mass.) was added to dry faeces as a recovery standard. Methyl esters were added to the faeces and the counts per minute (CPM) in fractions Band C were determined and compared with the total CPM added to the faeces. In both cases, at least 95% of the activity was recovered in its proper fraction. The sum of neutral steroid concentrations from B and C was at least 94% of the concentration of neutral steroids determined by direct saponification (A). Concentrations of free and esterified neutral steroids in the faeces of 25 North American and 28 South African subjects were determined with this method.

Statistical Analysis

Statistical significance of differences between median neutral steroid concentrations was determined with the Mann-Whitney U-test (2-tailed). Percentage esterification values were determined for each compound in each
individual. A median of these values was determined in each population for each compound. Statistical comparisons were made with the Mann-Whitney U-test (2-tailed). The difference in distribution of cholesterol conversion patterns was analyzed with the use of the chi-square test. Differences were considered to be statistically significant if $P<0.05$ for the 2-tailed test.

RESULTS

The North American Whites excreted higher concentrations of all of the animal steroids than did the South African Blacks (Table I). With the exception of ethylcoprostanol, which had a higher concentration in the faeces of the Blacks, there were no significant differences in plant steroid excretion (Table II). This comparison, however, was complicated by the fact that a large proportion of the neutral steroids in the faeces of the Blacks consisted of esterified rather than free (non-esterified) neutral steroids, whereas only a small proportion of the neutral steroids in the faeces of the North Americans was esterified. A comparison of free neutral steroids in the faeces of 25 of the North Americans and 28 of the Blacks, chosen at random from the populations reported in Tables I and II, is shown in Table III. The significance of the differences between the two populations in cholesterol, coprostanol and sitosterol increased when concentrations of free rather than total (free plus esterified) neutral steroids were compared. Comparison of coprostanone concentrations was not affected since coprostanone cannot exist as an ester.

Median percentage esterification values for cholesterol, coprostanol, sitosterol and ethylcoprostanol are shown in Table IV. Percentage esterifications of all four of these compounds were significantly higher in the faeces of the Blacks. Esterification of cholesterol and sitosterol was lower than that of their corresponding coprostanols in the faeces of the Blacks, but this was statistically significant only in the case of cholesterol. No significant difference in esterification of these compounds was found in

**TABLE I. MEDIAN CONCENTRATIONS* (mg/g DRY FAECES) OF NEUTRAL STEROIDS OF ANIMAL ORIGIN IN THE FAECES OF 98 NORTH AMERICAN WHITES (HIGH RISK FOR COLONIC CANCER) AND 84 RURAL SOUTH AFRICAN BLACKS (LOW RISK FOR COLONIC CANCER)**

<table>
<thead>
<tr>
<th>Number of subjects</th>
<th>Cholesterol</th>
<th>Coprostanol</th>
<th>Coprostanone</th>
<th>Total animal steroids</th>
</tr>
</thead>
<tbody>
<tr>
<td>North Americans</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>98</td>
<td>4,9</td>
<td>13,9</td>
<td>1,6</td>
<td>20,4</td>
</tr>
<tr>
<td>South Africans</td>
<td>1,8</td>
<td>10,3</td>
<td>0,8</td>
<td>12,9</td>
</tr>
</tbody>
</table>

* Determined by Mann-Whitney U-test (2-tailed).

**TABLE II. MEDIAN CONCENTRATIONS* (mg/g DRY FAECES) OF NEUTRAL STEROIDS OF PLANT ORIGIN IN THE FAECES OF 98 NORTH AMERICAN WHITES AND 84 RURAL SOUTH AFRICAN BLACKS**

<table>
<thead>
<tr>
<th>Number of subjects</th>
<th>Sitosterol</th>
<th>Ethylcoprostanol</th>
<th>Ethylcoprostanone</th>
<th>Campesterol</th>
<th>Methylcoprostanol</th>
<th>Methylcoprostanone</th>
<th>Total plant steroids</th>
</tr>
</thead>
<tbody>
<tr>
<td>North Americans</td>
<td>98</td>
<td>1,8</td>
<td>5,5</td>
<td>0,2</td>
<td>0,1</td>
<td>2,2</td>
<td>10,2</td>
</tr>
<tr>
<td>South Africans</td>
<td>84</td>
<td>1,7</td>
<td>6,8</td>
<td>0,2</td>
<td>0,1</td>
<td>2,7</td>
<td>11,2</td>
</tr>
</tbody>
</table>

* Determined by Mann-Whitney U-test (2-tailed).

**TABLE III. MEDIAN CONCENTRATIONS (mg/g DRY FAECES) OF MAJOR FREE (NON-ESTERIFIED) AND TOTAL (FREE PLUS ESTERIFIED) NEUTRAL STEROIDS IN THE FAECES OF 25 NORTH AMERICAN WHITES AND 28 RURAL SOUTH AFRICAN BLACKS CHOSEN AT RANDOM FROM POPULATIONS IN TABLE I**

<table>
<thead>
<tr>
<th>Number of subjects</th>
<th>Free neutral steroids</th>
<th>Total neutral steroids</th>
</tr>
</thead>
<tbody>
<tr>
<td>North Americans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>South Africans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>3,9</td>
<td>1,2</td>
</tr>
<tr>
<td>Coprostanol</td>
<td>9,4</td>
<td>4,9</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>1,5</td>
<td>1,1</td>
</tr>
<tr>
<td>Ethylcoprostanol</td>
<td>3,9</td>
<td>4,4</td>
</tr>
</tbody>
</table>

* Determined by Mann-Whitney U-test (2-tailed).

NS = not significant ($P>0.05$).
the faeces of the North Americans. In the faeces of both the Blacks and the North Americans, the following long-chain fatty acids were detected in the fraction containing the cholesterol esters: myristate (C14), palmitate (C16), stearate (C18), oleate (C18:1) and linoleate (C18:2).

The two populations also differed considerably in the distribution of cholesterol conversion patterns (Table V). The North American distribution was bimodal; in approximately 25% of the population less than 50% of the available cholesterol was converted to coprostanol (low conversion pattern). The South African distribution, however, was unimodal; in all but 4% of the Blacks at least 50% of the available cholesterol was converted to coprostanol and coprostanone (high conversion pattern). This difference was significant at the P<0.001 level. No statistically significant correlation between extent of esterification and extent of cholesterol conversion was seen in either population.

A comparison of groups within the North American population revealed no statistically significant age- or sex-associated differences in either neutral steroid concentrations or conversion patterns. This population of North Americans did not differ significantly in any way from the population of older North Americans studied previously. Similarly, within the South African population, it is important to note that no significant differences associated with age, sex or geographical location were found. This finding vitiates criticism over the differences in age of the two groups studied.

The mean percentage of water in the faeces of the Blacks was 73.5%, compared with 69.3% in the faeces of the North Americans. This difference was not significant. Thus, comparison of neutral steroid concentrations based on wet weight rather than on dry weight gave significant differences comparable to those shown in Tables I - III.

**DISCUSSION**

Differences in neutral steroid excretion between the high-risk North Americans and the low-risk Blacks occurred mainly in the neutral steroids of animal origin. Concentrations of cholesterol, coprostanol and coprostanone were much higher in the faeces of the North Americans than in the faeces of the Blacks. Although these differences were statistically significant, they were not nearly as great as the threefold differences reported by other workers.

The neutral steroids of plant origin, which were not determined in earlier surveys, accounted for approximately one-third of the total neutral steroids in the faeces of both populations. Except for ethylcoprostanol which was excreted in higher concentrations by the Blacks, no significant differences in plant steroid excretion were found. Drasar and Hill have suggested that some bacterial metabolites of cholesterol might be carcinogenic. Since plant steroids comprise an appreciable fraction of neutral steroids in the colon and since at least some bacterial enzyme systems that utilize cholesterol can also utilize sitosterol and campesterol as substrates, plant steroids as well as cholesterol should be considered as possible substrates in experiments of this type. If an organism which could produce carcinogens or co-carcinogens from animal steroids in vitro could also produce these compounds from plant steroids, serious questions would be raised about the significance of such reactions in determining risk in vivo.

The two populations also differed in the chemical state of their neutral steroids. Esterification of neutral steroids was much more extensive in the faeces of the Blacks than in the faeces of the North Americans. Because of this, the significance of the differences between populations was different when free (non-esterified) rather than total (esterified plus non-esterified) neutral steroid concentrations were compared. Thus, in future surveys of neutral steroid excretion by different populations, the extent of esterification in the faeces of each population should be determined.
In an earlier study of the esterification of neutral steroids in the faeces of 7 North Americans, Rosenfeld and Hellman found that 1-16% of the cholesterol, coprostanol and sitosterol was esterified. The results of the present study for a North American population are consistent with these values. The Blacks, however, had a significantly higher proportion of their faecal neutral steroids esterified, and this may indicate differences in bacterial metabolism of these compounds. Eysen et al. have reported that cholesterol-converting bacteria from a rat's caecum did not convert esterified cholesterol to coprostanol in vitro. However, most of the cholesterol in the intestinal tracts of the Blacks was reduced to coprostanol which was highly esterified. Accordingly, it is possible that esterification occurred after reduction of cholesterol in the colon and that the esterification reaction was carried out by components of the colonic flora. Esterification of neutral steroids or other hydroxylated compounds with fatty acids would make these substances less soluble. If reduction in solubility enhances binding of harmful substances to fibre, as has been suggested, this type of reaction might have a protective function.

Differences between the two populations were also seen when distribution of cholesterol conversion patterns was compared. The Black population was relatively homogeneous; nearly all of the Blacks were high converters of cholesterol to coprostanol and coprostanone.

By contrast, the North American population consisted of two distinct subpopulations: high converters of cholesterol and low converters of cholesterol. These results do not support the hypothesis of Hill et al. and others that extensive metabolism of cholesterol by intestinal organisms is associated with a high risk for cancer of the colon.

The existence of a sizeable minority of low converters in the North American population indicates that within this population there is considerable variation in metabolism of cholesterol by intestinal organisms. This variation in metabolism could be the result of differences in the chemical environment of the colon, possibly related to diet, which affect bacterial activity.

Thus, neutral steroids in the faeces of the low-risk South Africans and high-risk North Americans differ not only in concentration, but also in chemical state and availability for bacterial metabolism. Neutral steroids comprise 2-3% of the total dry weight of the faeces. Accordingly, the differences found between our high- and low-risk populations indicate that there may be differences in a significant component of the colonic environment of these two populations.

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REFERENCES