Biochemical and Clinical Effects of Selenium on Dimethylhydrazine-induced Colon Cancer in Rats

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ABSTRACT

The biochemical and clinical effects of selenium (Na$_2$SeO$_3$) on 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis in male Sprague-Dawley rats are presented. A 4-ppm selenium supplement to the drinking water was provided before, during, and after 20 weekly injections of 20 mg DMH per kg body weight. Immediately after the 20th DMH injection, part of the rats were sacrificed. The incidences of colon tumors in groups provided selenium before DMH, before and during DMH, and only during DMH treatment were reduced to 39, 43, and 36%, respectively. The incidence in the DMH-only control group was 63%. Other rats in all treated and control groups were maintained up to 5 months post-DMH treatment. At 10-week intervals throughout the study, selected blood and tissue components were analyzed. The following hematological changes correlated with DMH treatment. (a) Serum glutamic oxalacetic transaminase increased 2-fold (normal, 66 ± 14 g/dl). (b) Serum alkaline phosphatase increased 24% (normal, 166 ± 56 units/liter). (c) Serum protein decreased 14% (normal, 6.77 ± 0.48 g/dl). (d) White blood count increased 2- to 3-fold (normal, 7.7 ± 2.7 × 10$^3$/cu mm). And (e) hemoglobin decreased 6% (normal, 18.1 ± 1.3 g/dl). The magnitude of these changes varies with each selenium treatment group and with each 10-week analysis period. Provision of 4 ppm selenium doubled both liver and blood selenium levels compared to unsupplemented controls. The effects of selenium and DMH treatments on glutathione peroxidase and β-glucuronidase activities and on salic acid are presented. Possible mechanisms by which selenium protects against DMH-induced neoplasia are discussed.

INTRODUCTION

Previous studies have demonstrated selenium inhibition of colon, liver, skin, and mammary gland carcinogenesis. Using the male Sprague-Dawley rat model, selenium inhibited the colon tumor incidence in MAM-treated rats and reduced the total number of colon tumors in MAM-treated rats (14, 17). Selenium inhibited 2-acetylaminofluorene (25) and 3'-methyl-4-dimethylaminoazobenzene (11) hepatocarcinogenesis. The hepatic tumor incidence induced by 3'-methyl-4-dimethylaminoazobenzene was reduced by inorganic selenium (Na$_2$SeO$_3$) and by organic selenium (selenium yeast) supplements to the drinking water and diet, respectively. Reports from other laboratories have noted that selenium supplements decreased the incidence of hepatic tumors induced by 3'-methyl-4-dimethylaminoazobenzene (4) and 2-acetylaminofluorene (12) and of skin (31) and mammary tumors (13) induced by 7,12-dimethylbenz(a)anthracene. Selenium added to the drinking water reduced the incidence of spontaneous mammary tumors in C3H/St mice (29).

Epidemiological studies illustrate an increased incidence of colorectal, breast, and other cancers in humans in geographic regions where selenium is deficient (19, 33). Lower blood selenium levels in patients with gastrointestinal cancers have been observed in several clinical studies (1, 32). In experimental systems, supplements of selenium reduced the incidence of colon, breast, and liver cancers as cited above. In additional studies, we have reported the comparative reduction in DMH-induced colon tumors by supplements of selenium to drinking water, ascorbic acid to the diet, and butylated hydroxytoluene to the diet (16). In this study, selenium is provided before, during, and after DMH treatment. The "parameters" evaluated throughout include the CBC, serum protein, alkaline phosphatase, and SGOT. Liver selenium and GSH, a selenium-dependent enzyme, are correlated with microscopic pathology, SGOT, and alkaline phosphatase levels to insure that the selenium level added to the drinking water is not toxic. The colonic β-glucuronidase activity is assayed as a possible indicator of tumor development. The potential preventive and therapeutic attributes of selenium in colon cancer and the possible mechanism of action of selenium are discussed.

MATERIALS AND METHODS

Weanling male Sprague-Dawley rats purchased from SASCO (Omaha, Nebr.) were quarantined, randomized into treatment and control groups, and earmarked. Animals were housed 5/cage in plastic cages containing granular cellulose bedding (Bed-o'-cobs; Anderson Mills, Mony, Ohio) in temperature- and humidity-controlled rooms. A basal diet of Wayne Lab-Blox was provided ad libitum. Stock solutions (400 ppm) of selenium (Na$_2$SeO$_3$; purchased from Alfa Products, Ventrón Corp., Danvers, Mass.) were prepared weekly, diluted to 4-ppm selenium concentrations, and provided ad libitum to appropriate groups for defined time intervals during the study outlined below. The Wayne diet varied in composition between 0.1 and 0.5 ppm selenium and the drinking water contains less than 10 ppb selenium. According to our assay, the Wayne diet used in this study was 0.183 ppm selenium. Hence, our 4-ppm Se supplement was 22-fold higher than the dietary level in untreated controls. Rats treated with carcinogen received weekly s.c. injections of 20 mg of DMH (sym-dimethylhydrazine dichloride; 97% purchased from Aldrich Chemical Co. Inc., Milwaukee, Wis.) per kg body weight for 20 weeks. Body weights and survival were monitored throughout. At either death or scheduled sacrifice, complete necropsies were performed for evaluation of gross and microscopic pathology. Colons were dissected at the cecal and rectal junction and cut longitudinally. One half was preserved in formalin and hematoxylin and eosin stained for microscopic pathology. The other half of the colon was quick frozen on dry ice or rinsed in...
water and immediately scraped with a microscope slide for colon mucosa assays such as β-glucuronidase (27).

For clinical analysis and CBC, animals were anesthetized by CO₂ inhalation and exsanguinated by heart puncture. SGOT, alkaline phosphatase, and protein were determined on the Polimak Model 900 automated batch analyzer (P. M. America, Inc., Sacramento, Calif.). For SGOT, the AST (glutamic oxalacetic transaminase) Uni-UV diagnostic kit purchased from Sclavo, Inc. (Wayne, N. J.) was used. This is an automated kinetic assay in which the rate of NADH oxidation is proportional to the SGOT activity. For the alkaline phosphatase assay, the ALP-cinet diagnostic kit purchased from Sclavo, Inc., was used. This automated procedure uses p-nitrophenolphosphate and is an end point assay for p-nitrophenolate. The reagent kit for total protein was purchased from Sciavo, Inc. (Wayne, N. J.) was used. This is an automated batch analyzer (P. M. America, Inc., Sacramento, Calif.). For confirmation, the manual assay of Lowry et al. (26) for alkaline phosphatase. The CBC was determined on the Coulter Model ZF-5 and the hemoglobinometer both from Coulter Electronics, Inc. These analyses were carried out on freshly drawn whole blood or serum.

Sections of liver removed at necropsy were quick frozen on dry ice and stored frozen until homogenized and assayed. Selected manual assays were carried out on blood, liver, or colonic scrapedings. Liver and blood levels of selenium were measured by the spectrophotometric method of Watkinson (35) using an Amino-Bowman spectrophotometer. GSHP was assayed according to Burk et al. (2) using both hydrogen peroxide and cumene hydroperoxide as substrates. Other manual assays were for β-glucuronidase (27), sialic acid (34), and acid phosphatase (18). The Gillford Model 250 UV-VIS spectrophotometer was used for these assays. Linear regression and other statistics were calculated as described by Colton (5).

All rats were initiated on the study at 8 weeks of age. All DMH injections were given between 21 (first injection) and 40 (last injection) weeks of age. Relative to DMH treatments, supplemental selenium was provided in the drinking water for designated periods (weeks of age) either before, during, after, or combinations thereof. The experimental groups were as follows: Group 1, selenium given only during DMH treatment (21 to 40 weeks of age); Group 2, selenium given only for 13 weeks before DMH treatment (8 to 21 weeks of age); Group 3, selenium given for 13 weeks before and during DMH treatment (8 to 40 weeks of age); Group 4, selenium given only DMH treatment (41 weeks of age to death); and Group 5, selenium given during and after DMH treatment (21 weeks of age to death). The control groups were as follows: Group 6, selenium only (8 weeks of age to death); Group 9, selenium only before DMH had better overall survival than other selenium- and DMH-treated groups (Groups 3 to 5).

Table 1 also shows the body weight of experimental groups at 10-week intervals. Initially, all groups were weight matched at 106 ± 12 g/rat. The selenium only control (Group 6) weight gain at 61 weeks of age was slightly less than the untreated control, but with the overlap of standard deviation between these 2 groups this difference was not significant. During the 20 weeks of DMH treatment (Table 1, 21 to 41 weeks of age), the selenium only and untreated control groups gained approximately 67 g, the DMH only groups gained 45 g, and the DMH and selenium given together groups gained 4 g, suggesting a possible antagonism between DMH and selenium. By comparison, provision of selenium before DMH (Group 2) resulted in a 47-g weight gain, implying partial protection by selenium against DMH. Over the weeks following the last DMH injection (41 to 61 weeks of age), the weight responses were as follows: Group 2, +9 g; Group 3, +16 g; Group 4, -57 g; Group 5, +9 g; and Group 9, -9 g. Early and prolonged treatment with selenium protected against weight loss. Provision of selenium only after DMH treatment (Group 4, -57 g) was detrimental causing considerable weight loss.

Colon Tumors. For colon tumors, Table 2 shows the incidence, mean diameter, total number per group, and number of controls, only the data for Group 6 are presented in the tables and text. When selenium supplements were not provided, tap water was available ad libitum.

### RESULTS

#### Survival and Growth

Table 1 presents the survival and body weight for treatment and control groups at 10-week intervals through 61 weeks of age. The 4-ppm selenium supplement had no effect on survival. At 61 weeks of age, the survival of the selenium only control (Group 6) and of the untreated control (Group 10) was equivalent, 98% and 96%, respectively. In contrast, the survival of all DMH-treated groups was less than 50% at 61 weeks of age. At 41 weeks of age, the survival of all groups was 96% or greater. This was 1 week following the 20th DMH injection. Ten and 20 weeks following DMH treatments, the survival rate was markedly reduced compared to selenium only and untreated control groups. The survival of Group 5 rats given selenium during and after DMH was poorest and most notably different from all other groups (67% at 51 weeks and 30% at 61 weeks). The Group 2 rats provided selenium only before DMH had better overall survival than other selenium- and DMH-treated groups (Groups 3 to 5).

Table 1 also shows the body weight of experimental groups at 10-week intervals. Initially, all groups were weight matched at 106 ± 12 g/rat. The selenium only control (Group 6) weight gain at 61 weeks of age was slightly less than the untreated control, but with the overlap of standard deviation between these 2 groups this difference was not significant. During the 20 weeks of DMH treatment (Table 1, 21 to 41 weeks of age), the selenium only and untreated control groups gained approximately 67 g, the DMH only groups gained 45 g, and the DMH and selenium given together groups gained 4 g, suggesting a possible antagonism between DMH and selenium. By comparison, provision of selenium before DMH (Group 2) resulted in a 47-g weight gain, implying partial protection by selenium against DMH. Over the weeks following the last DMH injection (41 to 61 weeks of age), the weight responses were as follows: Group 2, -9 g; Group 3, +16 g; Group 4, -57 g; Group 5, +9 g; and Group 9, -9 g. Early and prolonged treatment with selenium protected against weight loss. Provision of selenium only after DMH treatment (Group 4, -57 g) was detrimental causing considerable weight loss.

### Colon Tumors

For colon tumors, Table 2 shows the incidence, mean diameter, total number per group, and number of

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>% of survival</th>
<th>BW (g)</th>
<th>% of survival</th>
<th>BW (g)</th>
<th>% of survival</th>
<th>BW (g)</th>
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<tr>
<td></td>
<td></td>
<td>21 wk</td>
<td>31 wk</td>
<td>41 wk</td>
<td>51 wk</td>
<td>61 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>DMH + selenium</td>
<td>100 (80)c</td>
<td>458 ± 40</td>
<td>97</td>
<td>454 ± 41</td>
<td>97</td>
<td>462 ± 49</td>
<td>83</td>
<td>531 ± 57</td>
<td>43</td>
<td>480 ± 71</td>
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<td>2</td>
<td>Selenium before DMH</td>
<td>100 (120)</td>
<td>442 ± 42</td>
<td>100</td>
<td>476 ± 49</td>
<td>97</td>
<td>498 ± 51</td>
<td>83</td>
<td>513 ± 53</td>
<td>36</td>
<td>483 ± 63</td>
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<tr>
<td>3</td>
<td>Selenium before and during DMH</td>
<td>98 (120)</td>
<td>435 ± 42</td>
<td>98</td>
<td>453 ± 47</td>
<td>96</td>
<td>467 ± 47</td>
<td>76</td>
<td>513 ± 53</td>
<td>36</td>
<td>483 ± 63</td>
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<td>100 (120)</td>
<td>451 ± 45</td>
<td>100</td>
<td>476 ± 45</td>
<td>97</td>
<td>493 ± 48</td>
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<td>476 ± 60</td>
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<td>100</td>
<td>453 ± 49</td>
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<td>476 ± 54</td>
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<td>482 ± 63</td>
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<td>441 ± 42</td>
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<td>465 ± 45</td>
<td>96</td>
<td>506 ± 45</td>
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<td>521 ± 49</td>
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<td>533 ± 50</td>
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<td>443 ± 45</td>
<td>100</td>
<td>470 ± 44</td>
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<td>488 ± 42</td>
<td>86</td>
<td>526 ± 51</td>
<td>47</td>
<td>517 ± 67</td>
</tr>
<tr>
<td>8</td>
<td>Untreated control</td>
<td>100 (80)</td>
<td>461 ± 42</td>
<td>98</td>
<td>508 ± 48</td>
<td>98</td>
<td>530 ± 53</td>
<td>97</td>
<td>572 ± 52</td>
<td>96</td>
<td>580 ± 54</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, initial number of rats per group. Mean ± S.D.
As indicated in Table 2, the incidence and number of tumors increased with time. The mean diameter of tumors also increased, with mean values ranging from 3.2 mm at the start of DMH treatment to 7.0 mm at the end of the 61-week period. The incidence of tumors was highest in the untreated control group, with 36% of rats developing tumors by 61 weeks of age. In contrast, rats given selenium before and during DMH treatment (Group 3) had the lowest tumor incidence, with only 36% developing tumors by the same time point.

The data in Table 2 also show that selenium supplementation before DMH treatment provided the greatest protection against long-term DMH-induced tumorigenesis. Provision of selenium before, during, and after carcinogen administration reduced the colon tumor incidence to 43 and 39%, respectively, compared to the DMH only control of 63% (Table 2, 41 weeks old). Between 51 and 61 weeks of age, the tumor incidence in the DMH only control group increased to 70%, while selenium supplementation before and during DMH treatment reduced the tumor incidence to 50% (Groups 3 and 4).

The majority of DMH-induced colon tumors were roughly spherical and symmetrical. The mean diameter and tumor volume are only crude estimates but do indicate the relative degree of tumor burden in various treatment groups. In general, the mean diameter increased with time from means of 3.2 (31 weeks of age; tenth DMH injection) to 7.0 mm (51 to 61 weeks of age) and 61 weeks of age or following 10 to 20 weeks from the last DMH injection.

Considering all of the data in Table 2, 3 observations are itemized for treatment and control groups midway through the course of 20 DMH injections (41 weeks), and 10 to 20 weeks following the last DMH injection (51 to 61 weeks). Tumors were verified histopathologically to be adenocarcinomas and villous adenomas of the colon. Colon tumors often developed out of Peyer's patches approximately 5 cm from the cecal junction and 2 to 5 cm from the rectal junction.

Immediately after the 20th DMH injection, 30 rats were sacrificed at 41 weeks of age in each experimental group and 10 rats in the untreated control. Provision of selenium simultaneously with DMH treatment reduced the colon tumor incidence from 63% (Group 9; DMH only control; 41 weeks old) to 36% (Group 1, 41 weeks old). This 50% reduction confirmed earlier reports from this laboratory (14, 16, 17).

Selenium provided before and during (Group 3) as well as only before (Group 2) DMH treatment also reduced the colon tumor incidence to 43 and 39%, respectively, compared to the DMH only control of 63% (Table 2, 41 weeks old). Between 51 and 61 weeks of age or following 10 to 20 weeks from the last DMH injection, all DMH-treated groups developed an approximate 90% colon tumor incidence regardless of whether the selenium provision was before, during, and/or after carcinogen administration.

The majority of DMH-induced colon tumors were roughly spherical and symmetrical. The mean diameter and tumor volume are only crude estimates but do indicate the relative degree of tumor burden in various treatment groups. In general, the mean diameter increased with time from means of 3.2 (31 weeks of age; tenth DMH injection) to 4.5 (41 weeks of age; 20th DMH injection) and to 7.0 mm (51 to 61 weeks of age; 10 to 20 weeks following the 20th DMH injection).

Considering all of the data in Table 2, 3 observations are outstanding. (a) At 31 weeks, Group 2 rats provided selenium only before DMH had the lowest tumor incidence, lowest mean tumor diameter, and lowest number of tumors per tumor-bearing animal. (b) At 41 weeks, Group 1 rats provided selenium only during DMH treatment had the smallest tumor diameter and lowest tumor incidence. And (c) between 51 and 61 weeks, Group 5 rats given selenium during and after DMH had the smallest tumor diameter and lowest number of tumors per tumor-bearing animal among selenium- and DMH-treated groups. In summary, selenium supplementation before DMH treatment protected against the short-term effects of DMH, while selenium given during and after DMH offered protection against long-term DMH-induced tumorogenesis. Provision of selenium only after DMH treatment (Group 4) appeared to enhance the development of new colon tumors since at 41 weeks of age there were 1.6 tumors/tumor-bearing animal and this increased to 3.5 tumors/tumor-bearing animal between 51 and 61 weeks of age.

Extracolonic tumors were not apparent in animals sacrificed at intervals up to 41 weeks of age corresponding to the 20th DMH injection. After this time, however, tumors were observed in the small intestine (40%), pancreas (35%), Zimbal gland (38%), and cecum (2%) with the incidence unchanged in selenium-supplemented animals compared to DMH only controls. Presumably, these tumors are primary tumors at extracolic sites but may, in fact, represent metastasis. In all animals having extracolonic tumors, there were also colon tumors.

CBC. The CBC includes RBC, WBC, hematocrit, hemoglobin, and calculated mean cell volume run on individual animals. Table 3 shows the CBC for all groups at 41 weeks of age. Since the CBC was unchanged during tumor initiation and early development compared to selenium only and untreated control groups, only the data from selected experimental groups at 41, 56, and 64 weeks of age are presented to illustrate alterations associated with DMH treatment. The mean calculated cell volume did not change with increased age or selenium with or without DMH treatment and is therefore reported only at 41 weeks of age.

The WBC increased 2- to 3-fold with DMH treatment. The increase observed in Groups 2 to 5 and 9 (DMH only control) at 56 weeks was returned closer to the normal WBC (7.7 x 10^3/cu mm) at 64 weeks of age in Groups 2, 4, and 5. This compares favorably with the earlier report in which selenocystine treatment reduced total leukocyte counts providing therapeutic control of leukemia patients (39).

Comparing the hematocrit in selenium only and untreated control (Table 3, Group 10) groups, there is a decrease to similar levels with increased age. The decrease is also present in DMH-treated groups (Groups 2 to 5) at 64 weeks of age.
Selenium Inhibition of DMH-induced Colon Cancer

Hence, this CBC parameter change is an age-related phenomenon that does not independently reflect either selenium or DMH treatment.

The hemoglobin decreases with increased age in selenium only (Table 3, Group 6), untreated control (Group 10), and DMH only (Group 9) groups between the ages of 41 to 64 weeks. The decrease is greatest in DMH with or without selenium treatment groups and is sustained with time. The reduction in hemoglobin is not restored to normal by selenium supplementation either before or after DMH treatment.

Serum Protein, SGOT, and Alkaline Phosphatase. According to Table 4, there is an apparent reduction in the serum protein level with age and a greater reduction with DMH treatment. Between 41 and 61 weeks of age, the mean serum protein for Groups 2 to 5 declines 0.93 g/dl, the selenium only Group 6 rises 0.17 g/dl, and the DMH only and untreated controls decline 1.09 and 0.47 g/dl, respectively. Not only is the serum protein level age related but also this parameter correlates with DMH treatment.

The serum SGOT level was determined on individual animals sacrificed at increasing time intervals before, during, and after DMH with and without selenium treatment. The SGOT activity at 41, 51, and 61 weeks of age is expressed in Table 4. Large standard deviations were characteristic throughout. Nevertheless, the SGOT increased markedly in DMH- and selenium-treated Groups 2, 4, and 5 with increased age and progressive tumor development.

Also illustrated in Table 4 are the serum alkaline phosphatase levels in DMH with and without selenium and control animals at 41, 51, and 61 weeks of age. As with SGOT, the standard deviations are large in each group and increase with increased tumor burden reflecting individual animal variation within the groups. The baseline normal level was 166 ± 56 units/liter and remained as such within standard deviation up to 61 weeks of age in selenium only controls (Table 4, Group 6) and the untreated control. All DMH-treated groups had markedly increased alkaline phosphatase levels compared to the untreated control at each 10-week interval assayed. The selenium only control (Table 4, Group 6) had a higher serum alkaline phosphatase than the untreated control but lower than DMH-treated groups.

For each clinical test, the proposed increase or decrease in response to treatment evolved from a comparative analysis of data obtained for treatment versus control groups with increase in age. This approach was taken because of the characteristically large variation among individual animals within a group. At a single time, assays were frequently not significantly different between treatment and control groups. It was therefore necessary to trace the history of individual animals throughout treatment from 8 to 64 weeks of age with respect to each assay (e.g., alkaline phosphatase). The comparison of changes occurring in one group with changes in another within an identical time frame gave rise to the selection of markers which appeared to correlate with colon tumor development and/or DMH treatment. For example, the alkaline phosphatase level at 41 weeks of age in Group 4 is 219 ± 109 units/liter and is not significantly increased over the untreated control Group 10 (127 ± 33 units/liter). The enzyme level in Table 4 for Group 4 (selenium after DMH) at 10-week intervals is as follows: 21 weeks, 197 ± 51 units/liter; 31 weeks, 204 ± 29 units/liter; 41 weeks, 219 ± 109 units/liter; 51 weeks, 246 ± 125 units/liter.
Effect of selenium on liver selenium and GSHP, serum alkaline phosphatase, and sialic acid and colon β-glucuronidase with DMH treatment of Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Protein (g/dl)</th>
<th>SGOT (unit/liter)</th>
<th>Alkaline phosphatase (unit/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>41 wk</td>
<td>51 wk</td>
<td>61 wk</td>
</tr>
<tr>
<td>1</td>
<td>DMH + selenium</td>
<td>6.17 ± 0.48a</td>
<td>71 ± 28</td>
<td>221 ± 93</td>
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<tr>
<td>2</td>
<td>Selenium before DMH</td>
<td>6.29 ± 0.66</td>
<td>78 ± 33</td>
<td>200 ± 85</td>
</tr>
<tr>
<td>3</td>
<td>Selenium before and during DMH</td>
<td>6.07 ± 0.38</td>
<td>65 ± 19</td>
<td>218 ± 84</td>
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<tr>
<td>4</td>
<td>Selenium after DMH</td>
<td>6.13 ± 0.66</td>
<td>76 ± 31</td>
<td>219 ± 109</td>
</tr>
<tr>
<td>5</td>
<td>Selenium during and after DMH</td>
<td>6.17 ± 0.48</td>
<td>71 ± 28</td>
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</tr>
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<td>6</td>
<td>Selenium only control</td>
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<td>55 ± 14</td>
<td>165 ± 73</td>
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<td>DMH only</td>
<td>6.13 ± 0.38</td>
<td>76 ± 31</td>
<td>219 ± 109</td>
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<tr>
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<td>Untreated control</td>
<td>6.27 ± 0.66</td>
<td>70 ± 27</td>
<td>127 ± 33</td>
</tr>
<tr>
<td>9</td>
<td>Baseline</td>
<td>6.77 ± 0.48</td>
<td>68 ± 14</td>
<td>166 ± 56</td>
</tr>
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</table>

a Mean ± S.D.

Effect of selenium on liver selenium and GSHP, serum alkaline phosphatase, and sialic acid and colon β-glucuronidase with DMH treatment of Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Liver GSHP (μM NADP/mg protein)</th>
<th>Liver selenium (μg selenium/g wet wt)</th>
<th>Serum acid phosphatase (μM NADPH hydrolyzed x 10^-3/mg protein/20 min)</th>
<th>Sialic acid (μM NANA hydrolyzed/mg protein/30 min)</th>
<th>Colon β-glucuronidase (mm phenolphthalein glucuronide hydrolyzed/mg protein/hr)</th>
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<tr>
<td>1</td>
<td>DMH + selenium</td>
<td>60.8 ± 10.6a</td>
<td>2.36 (21)</td>
<td>26.8 ± 4.4 (31)</td>
<td>7.1 (31)</td>
<td>0.115 (41)</td>
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<tr>
<td>2</td>
<td>Selenium before DMH</td>
<td>58.2 ± 2.5</td>
<td>3.24 (41)</td>
<td>23.5 ± 4.6 (38)</td>
<td>12.3 (31)</td>
<td>0.079 (41)</td>
</tr>
<tr>
<td>3</td>
<td>Selenium before and during DMH</td>
<td>65.2 ± 14.0a</td>
<td>2.65 (21)</td>
<td>23.4 ± 6.0 (31)</td>
<td>7.7 (31)</td>
<td>0.079 (41)</td>
</tr>
<tr>
<td>4</td>
<td>Selenium only</td>
<td>55.6 ± 9.0a</td>
<td>2.02 (41)</td>
<td>21.9 ± 1.9 (41)</td>
<td>6.7 (41)</td>
<td>0.079 (41)</td>
</tr>
<tr>
<td>5</td>
<td>DMH only</td>
<td>56.0 ± 6.0a</td>
<td>1.78 (51)</td>
<td>38.5 ± 7.3 (61)</td>
<td>8.9 (51)</td>
<td>0.079 (41)</td>
</tr>
<tr>
<td>6</td>
<td>Untreated control</td>
<td>52.7 ± 11.4a</td>
<td>2.50 (61)</td>
<td>40.4 ± 6.4 (61)</td>
<td>11.6 (31)</td>
<td>0.598 (41)</td>
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<td>7</td>
<td>Baseline</td>
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<td>0.702</td>
<td>28.7 ± 2.5 (41)</td>
<td>8.0 (41)</td>
<td>0.070 (41)</td>
</tr>
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a All assays at 31 weeks of age following 10 injections of DMH.
b NP, N-p-nitrophenolate; NANA, N-acetyleneuraminic acid.
c Serum assays on 3 to 6 rats; colon assays on pooled sample from 2 to 5 rats.

litter; 61 weeks, 254 ± 137 units/liter. The corresponding enzyme levels in the untreated control Group 10 are: 21 weeks, 197 ± 51 units/liter; 31 weeks, 178 ± 45 units/liter; 41 weeks, 127 ± 33 units/liter; 51 weeks, 202 ± 84 units/liter; and 61 weeks,110 ± 46 units/liter. Comparison of these and additional data at intermediate time intervals between 8 and 61 weeks of age suggests there is in fact an increase in alkaline phosphatase.

An alternate method of analysis was attempted to obtain a more direct correlation between tumors and each clinical response. This method was based on the observation that the colon tumor incidence through 41 weeks of age was less than 100% in DMH-treated groups. Each clinical test per group was subdivided into responses in animals with tumors versus responses in animals without tumors. There were no differences. This implied that early changes (initiation of DMH-induced tumors) which develop into gross and microscopically identifiable tumors had already occurred. This gave rise to clinical responses which precede morphological changes commonly used for pathological evaluation.

**Selenium and GSHP.** Using the spectrofluorometric method of Watkinson (35), both liver and blood selenium levels were measured at 41 weeks of age. The untreated control Group 6 had nearly 2-fold higher selenium than the selenium only control Group 6, which had 0.445 μg selenium per ml blood in the untreated control to 0.662 μg selenium per ml blood in the selenium only Group 6.
Liver obtained at necropsy of 41-week-old rats were quick frozen on dry ice and kept frozen until GSHP analysis. Immediately before analysis, the livers were homogenized and centrifuged, and the supernatant was assayed for GSHP activity (2). Using hydrogen peroxide and cumene hydroperoxide as substrates, the units of GSHP activity are expressed as μmol NADPH oxidized per mg protein. Table 5 illustrates the GSHP activity measured in corresponding animals with both substrates. The selenium only control exhibited the lowest activity (Group 6, 52.7 ± 11.4 units), the DMH- and selenium-treated Groups 1 to 3 an intermediate level (mean, 63.6 units), and the untreated control the highest level (107.9 ± 14.8 units). Similarly, the selenium-independent GSHP assayed with cumene hydroperoxide was lowest in the selenium only control, intermediate in selenium and DMH Groups 1 to 3, and highest in the untreated control. Having obtained these results with liver samples, pooled samples of colonic scrapings were assayed for the selenium-dependent GSHP using H₂O₂ as substrate. The selenium only-supplemented Group 6 was 5.39 ± 1.2 units and was higher than the DMH control Group 9 (4.66 ± 1.5 units). Again, the untreated control Group 10 was highest (5.76 ± 2.7 units) and the DMH and selenium Group 3 was at an intermediate level of 4.85 ± 0.9 units. These data are supplementary to Table 5. A possible interpretation of the GSHP data is presented in "Discussion."

Earlier, we reported the acute and chronic toxicity of selenium using survival, growth, clinical chemistry, and pathology as monitors (15). On the basis of these data, we selected 4 ppm selenium as the supplemental dose for these carcinogenesis studies. High (16- and 64-ppm) selenium stunted growth and increased the serum alkaline phosphatase and SGOT levels, while selenium supplements below 8 ppm had no effect on these parameters. Liver cell degeneration and necrosis, as well as bile duct proliferation, were observed in occasional untreated control and high-dose (16- and 64-ppm) selenium-treated rats. Neither pyknotic cells nor a large number of dividing cells accompanied this observation, thus denouncing premalignant tendencies related to high doses of selenium. The increase in SGOT in 16- and 64-ppm selenium-treated groups is in clinical agreement with damage to the parenchymal cells but is not observed in 4-ppm selenium-treated groups. None of the above monitors of selenium toxicity was observed in the 4-ppm selenium only control Group 6 through 69 weeks of age. Morphological, clinical, and biochemical changes are in large part due to DMH treatment but also to increased age and to the combined effects of selenium and DMH treatment.

β-Glucuronidase. Human and animal studies associate an increase in fecal bacterial β-glucuronidase with increased colon cancer or increased risk (10, 28). Using the same method (27) with an adjustment of the reaction mixture to pH 7.0 for the bacterial enzyme and to pH 4.5 for the colonic enzyme, we assayed both enzyme sources. The units of β-glucuronidase activity are expressed in Table 5 as nmol phenolphthalein glucuronide hydrolyzed per mg protein per hr. Both enzymes are lowest in the untreated control Group 10, increased in the selenium only control Group 6 and DMH only control Group 9, and appear to be highest in DMH and selenium Group 3 (Table 5). The colonic enzyme is approximately 5 to 10 times lower than the bacterial enzyme. Both sources of the enzyme were assayed at 41 weeks of age or 1 week following the 20th DMH injection. The colonic enzyme was again assayed 10 weeks later at 51 weeks of age.

Sialic Acid. If in fact an increase in mucopolysaccharides (containing sialic acid residues) in colon or serum were related to selenium and/or DMH treatment, supportive data from sialic acid assays were ascertained. The serum sialic acid determinations were after 10, 15, and 20 DMH injections and the colonic sialic acid determinations following 10 and 20 DMH injections. The sialic acid concentration is expressed in units as μmol N-acetylneuraminic acid hydrolyzed × 10⁻³ per mg protein per 30 min in Table 5. The sialic acid level is approximately 4-fold higher in the serum than in the colonic scrapings.

With DMH treatment and colon tumor induction, the serum sialic acid increased. The colon sialic acid showed no apparent change although these assays were conducted on pooled colon scrapings from 2 to 5 rats/assay. Whether or not selenium supplementation prevents as large an increase as observed in the DMH only controls will require more assays for clarification. The data in Table 5 indicate that the increase in serum sialic acid is an age-related phenomenon.

Acid Phosphatase. The serum acid phosphatase activity increases in prostatic carcinoma and multiple myeloma with concurrent changes in isozyme patterns (9). Determination of a possible correlation between colon carcinoma and the acid phosphatase was attempted. Table 5 shows the acid phosphatase expressed in units as μmol p-nitrophenyl phosphate hydrolyzed × 10⁻³ per mg protein per 20 min for treatment and control groups. Individual serum samples from 5 to 20 rats/group were assayed at each 10-week interval. The mean acid phosphatase in the untreated control Group 10 was 2.3 ± 0.23 units and was unchanged through 61 weeks of age. With selenium supplementation, the mean level of 1.76 ± 0.11 units (Table 5, Group 6) was lower and remained constant with increased age. All groups receiving DMH with or without the selenium supplement had higher acid phosphatase levels than the selenium only control but slightly less than the untreated control. The DMH and selenium treatment and colon tumor development appeared to be accompanied by a reduced acid phosphatase level at 41 weeks of age (last DMH injection) and at 51 weeks of age (10 weeks after the last DMH injection) in Groups 2 and 3 (Table 5). The increased acid phosphatase level in prostatic cancer and other diseases is commonly determined using β-glycerophosphate as substrate. The inverse response using the p-nitrophenyl phosphate synthetic substrate in this study possibly reflects different substrate specificities of the acid phosphatase and a possible means of distinguishing prostatic from colonic cancer.

DISCUSSION

In the foregoing study, many clinical and biochemical assays have been conducted during the induction and development of colon tumors. The effect of selenium supplementation before, during, and after DMH treatment on the parameters assayed has been tabulated at 10-week intervals or other selected time periods for brevity. One aim of this study was to obtain diagnostic and therapeutic markers of colon cancer. Such markers implicated by the data presented include increased WBC, reduced hemoglobin, increased SGOT and alkaline phosphatase, and perhaps decreased acid phosphatase. While each individual marker is not specific for colon cancer, the collective changes in all of these parameters appear to correlate with
DMH treatment, with colon tumor development, and with the effect of selenium on DMH-induced carcinogenesis.

A second aim of this study was to suggest a mechanism by which selenium inhibits DMH-induced neoplasia. Consideration is first given to the GSHP data reported, then to an interpretation of the β-glucuronidase data, and finally to plausible mechanisms of selenium inhibition.

Our initial intent in measuring GSHP activity was to evaluate the selenium status of the animals, anticipating that selenium-supplemented rats would have increased selenium-dependent enzyme activity. On the contrary, according to Table 5, the activity of both forms of the enzyme was lowest in the selenium only group, intermediate in the selenium- and DMH-treated groups, and highest in the untreated control. The selenium-dependent GSHP efficiently uses H₂O₂ as substrate, while the selenium-independent GSHP more efficiently uses cumene hydroperoxide as substrate (2, 23). The relative contributions of each enzyme to the total activity measured with each substrate is not known for the samples we assayed. Using H₂O₂, the greater range in activity was observed. Other inhibitors of carcinogen-induced neoplasia increase the glutathione S-transferase activity (3). Implicit to this observation is an increase in glutathione. Perhaps selenium induces an abundance of glutathione, and this in turn induces substrate inhibition yielding a decrease in GSHP activity in the selenium only-supplemented group. Alternatively, the glutathione substrate may be used more efficiently by the transferase having decreased substrate availability for the GSHP. In a report by Scott et al. (30), the supplementation of the diets of rats with increasing levels of selenium caused corresponding increases in the GSHP activity up to a point. At the highest level of selenium (5 ppm), the enzyme activity decreased. Perhaps a similar relationship is being observed in this study.

Epidemiological studies indicate that Americans consuming a mixed “Western” diet had higher levels of fecal bacterial β-glucuronidase than American vegetarians, Seventh Day Adventists, Chinese, or Japanese (28). Presumably a high beef diet is a risk factor altering bacterial enzymes responsible for the conversion of procarcinogens to proximal carcinogens. To test this experimentally, rats treated with DMH were administered a beef diet simulating a “Western” diet. A higher colon tumor incidence resulted. Supplementation of the beef diet with Lactobacillus acidophilus reduced the tumor incidence and the fecal bacterial β-glucuronidase (10). Since oral selenium reduced the colon tumor incidence in our studies, we assayed the bacterial β-glucuronidase to determine whether selenium reduced this enzyme activity as a suggested mechanism of action. We observed that the bacterial β-glucuronidase was higher in DMH-treated rats than untreated controls but was greatest in selenium- and DMH-treated animals. Hence, selenium inhibition of DMH-induced colon tumors could not be attributed to this enzyme. The increase in β-glucuronidase in the selenium only control perhaps reflects a response to other as yet unidentified glycoconjugates also recognized as suitable substrates by the hydrolytic enzyme. These substrates may be normal constituents of the mucous secretions in the colon but are perhaps in greater abundance at 41 weeks of age following prolonged exposure to the subtoxic selenium supplement.

Current data suggest that DMH is activated through the following sequence: DMH → azomethane → azoxymethane → MAM. Under physiological conditions, MAM is unstable and breaks down to form the strong alkylating agent, methyl diazonium hydroxide, and formaldehyde (6, 8). Laquer et al. (22) found the MAM glycoside (cycasin) to be a potent carcinogen in conventional rats but not in germ-free rats (22). From multiple bioassays of synthetic and natural metabolites, Laquer (20, 21) reported that the aglycone (MAM) produced the same effects in germ-free rats that the intact glucoside produced in conventional rats. Their data indicated that the intestinal bacteria possessed the β-glucosidase for liberation of the aglycone from the conjugate. Studies with inhibitors have increased our knowledge of the activation sequence for carcinogens as well as the mechanism and loci of inhibitor actions (36, 38). For example, disulfiram and related compounds that contain a carbon disulfide moiety inhibit DMH-induced neoplasia. The targets of disulfiram appear to be inhibition of the N-oxidation of azomethane and the hydroxylation of azoxy methane to MAM (7, 37). Pyrazole acts at other loci, the azoxy methane hydroxylase as well as the alcohol dehydrogenase activation of MAM (40). Although specific metabolites and enzymes indicated above were not assayed in the present study, selenium could similarly act at one or more of these loci causing inhibition of DMH-induced as well as MAM-induced neoplasia reported elsewhere (14).

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Selenium Inhibition of DMH-induced Colon Cancer


Biochemical and Clinical Effects of Selenium on Dimethylhydrazine-induced Colon Cancer in Rats

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