Effect of Butachlor on the Biosynthesis of Lipid, Protein, and Nucleic Acid in Three Isolated Plant Cells

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About one half of lipid synthesis was inhibited in Chlorella (46%), tobacco leaf cell (58%), and rice embryo cell (45%) when the isolated cell was incubated at 70 μM of butachlor for 4 hr. A thirty percent difference of unincorporated acetate pool size between 70 μM of butachlor treated (71%) and untreated (41%) tobacco leaf cells was found in lipid synthesis, but no significant difference was observed in that of Chlorella and rice embryo cells. Within 30 min incubation at 70 μM of butachlor, protein synthesis was inhibited by 79, 59, and 38% for Chlorella, tobacco leaf cells, and rice embryo cells, respectively, and the inhibition was increased by 92, 95 and 85%, respectively, at 8 hr. In contrast to untreated cells, there were 69, 62, and 39% larger pools of absorbed 14C-leucine after treatment of the three cell types with 70 μM of butachlor. However, total leucine uptake showed in treated cells was only 52, 37, and 51%, respectively, of that in control. Butachlor inhibited RNA synthesis at 70 μM, but at lower concentrations, even stimulation was found.

INTRODUCTION

Butachlor [N-(butoxymethyl)-2-chloro-N-(2,6-diethylphenyl)acetamide], a pre-emergence herbicide, is extensively used in paddy fields in Asia, including China, India, Japan, Korea, the Philippines and Taiwan. Butachlor is one of the chloroacetamide herbicides. Chloroacetamide herbicides such as alachlor [2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide], CDAA [2-chloro-N,N-di-2-propenylacetamide], metolachlor [2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)acetamide], and propachlor [2-chloro-N-(1-methylethyl)-N-phenylacetamide] are characterized as general growth inhibitor. They inhibit growth by preventing cell enlargement and mitotic entry. Chloroacetamides inhibit several metabolic processes but the primary site of action has not been identified. They were also found to inhibit anthocyanin synthesis. However, information concerning the mechanism of action for butachlor is still incomplete. Butachlor showed no effect on the germination of rice or barnyardgrass seeds but inhibited the growth of rice seedlings from the germination stage to the two-leaf stage. Butachlor applied at the early post-emergence stage would interfere the amino acid and protein synthesis of rice and barnyardgrass by inhibiting the activity of nitrate reductase but not glutamate dehydrogenase. Protein synthesis was inhibited by 50 μM of butachlor in both rice (Oryza sativa L.) and barnyardgrass (Echinochloa crusgalli L.) to a greater degree in roots than in shoots. The herbicide also inhibited protein synthesis in isolated leaf cells of red kidney bean (Phaseolus vulgaris L.). The present research was designed to study the effects of butachlor on the biosynthesis of lipid, protein, and nucleic acid in Chlorella, tobacco leaf cells, and rice embryo cells to add information on the mechanism of action of butachlor.
MATERIALS AND METHODS

1. Materials

Seeds of tobacco (Nicotiana tabacum cv. Wisconsin 38) and callus of rice (Oryza sativa cv. TN3) were obtained from the Department of Botany and Department of Agronomy, National Taiwan University, respectively. Chlorella (Chlorella penadosia CH-4263) cultures were provided by Refining and Manufacturing Research Center, China Petroleum Corp, Chiayi, Taiwan. Pure butachlor of 99.2% purity were obtained from Monsanto Co., St Louis, MO, U.S.A. Sodium 14C-acetate, L-[1-14C]leucine, and [2-14C]uracil were purchased from Amersham International plc, England, with specific activity of 2.07, 2.07 and 2.00 MBq/μmol, respectively.

2. Suspension Culture of Tobacco Leaf Cell and Isolation of Protoplast

Seeds of tobacco were soaked in 75% alcohol for 1 min and sterilized with 1% sodium hypochlorite solution for 15 min. After washing three times with deionized water, the seeds were cultured on MS medium in Petri dishes under 16 hr/day illumination with light intensity of 3000 lux at 25°C for five weeks. Tobacco mesophyll protoplasts were isolated by the method described by Huang & Chen. Three to four leaves from near the shoot apex were taken by using a sterile scalpel, sliced into strips of 1 mm breadth, and incubated in K medium supplemented with phytohormones and enzyme solution. The enzyme solution consisted of cellulase (10 g/l) and pectinase (2 g/l) (Onozuka R-10 and Macerozyme R-10, Yakult Honsha Co., Ltd., Japan). Incubation was performed for about 16 hr in the dark at 25°C. The medium was then filtered with a nylon-net filter (75 μm pore size) to remove the undecomposed scrap. Filtrate was centrifuged for 5 min (100 g), the upper protoplast suspension was pipetted to another tube and mixed with 15 ml of washing medium (W5 solution: 154 mM NaCl; 125 mM CaCl2·2H2O; 5 mM KCl; and 5 mM glucose; pH 5.6) and centrifuged. Protoplast (lower layer) was washed with the same washing medium in additional twice to remove enzyme solution, and then incubated in K medium in a population density of 10⁶ cells/ml for experimental use.

3. Suspension Culture of Rice Embryogenic Cell and Isolation of Protoplast

The embryogenic calli obtained from cultured immature embryos of rice seed was transplanted and cultured in a 125 ml flask containing 30 ml of MS liquid medium supplemented with 10 μM of 2,4-D on a shaker (120 rpm). Subculture was performed at every seven days. About 2 g of suspension cell was removed from the upper part of the culture with a sterile spatula and mixed in a Petri dish containing 10 ml of sterilized CPW 13 M salt solution (consists of following salts, in mg/l: KH₂PO₄ 27.2; KNO₃ 101; MgSO₄·5H₂O 246; KI 0.16; CuSO₄·5H₂O 0.025; CaCl₂·2H₂O 1480; mannitol 130; pH 5.8) supplemented with 1% cellulase RS (Yakult Honsha Co., Ltd.) and 0.1% pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Japan). After 9 hr incubation in the dark at 25°C, the medium was filtered (63 μm pore size) and the filtrate was centrifuged (600 rpm). The precipitant was washed with 10 ml of CPW 13 M salt solution, the protoplast was then incubated in protoplast culture medium (KM8P) in a population density of 10⁶ cells/ml for experimental use.

4. Experiment for the Effects of Butachlor on Metabolic Processes

The methods for studying the metabolic processes were essentially the same as those used by Francki et al. and Porter & Bartels. One milliliter of culture medium containing a population density of 10⁶ cells/ml was placed in the test tubes and butachlor was added to the concentrations of 0, 0.07, 0.7, 7, or 70 μM. Butachlor was diluted to give a final concentration of 1% ethanol in the incubation medium. Then, 18.5 kBq of sodium 14C-acetate, 14C-l-leucine, or 14C-uracil was added to the medium for determination of the effects of butachlor on lipid, protein, or RNA synthesis, respectively. The media were incubated at 25°C, then an aliquot of media (100 μl) were sampled from each concentration at 0.5, 1, 2, 4, and 8 hr. They were then filtered with Millipore (pore size 0.45 μm,
Satorious Co., West Germany) for protein and RNA or with glass fiber paper (GF-A, Whatman, England) for lipid. The cells on the filter were collected and placed in a 20 ml glass scintillation vials containing 10 ml of scintillation cocktail (Filter count, Packard Co., U.S.A.). The radioactivity of the samples was measured for 10 min using LS-1801 liquid scintillation system (Beckman). The amounts of radioactivity indicate the total amounts of uptake. On a separate study, the cells on the filter were washed thrice with 5 ml of 10% ice-cold trichloroacetic acid solution, extracted thrice with 5 ml of CHCl₃: MeOH (1: 1) for lipid or washed thrice with 80% EtOH for the protein and RNA synthesis experiment. One half milliliter of 30% H₂O₂ was added to the extracts or the filter, stood overnight at 50°C, and the radioactivity was measured. The amounts of radioactivity indicate the incorporated portions.

**RESULTS AND DISCUSSION**

Some reports suggest that chloroacetamides have little or no effect on lipid metabolism. Warmund et al. 18 reported that alachlor (8.2 μM) did not alter total lipid biosynthesis or fatty acid composition of sorghum (*Sorghum bicolor* L.). That metolachlor (10 μM) had no effect on acetate incorporation into lipids of

<p>| Table 1 Influence of butachlor concentration on the incorporation of sodium ¹⁴C-acetate in lipid synthesis of chlorella, tobacco leaf cell and rice embryo cell during different incubation periods. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Butachlor concentration (μM) | Chlorella | Tobacco leaf cell | Rice embryo cell |</p>
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<th>Inhib. b) (%)</th>
<th>Incorpor. a) (dpm)</th>
<th>Inhib. (%)</th>
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</table>

a) Acetate incorporated. Means within columns with similar letters are not significantly different at the 5% level by Duncan's multiple range test.

b) Inhibition percentage means the amounts of incorporated ¹⁴C-acetate reduced from free of butachlor to different concentrations compared with free of butachlor. A minus sign indicates stimulation instead of inhibition.
sorghum roots was also reported by Yenne & Hatzios. In conflict with this, Chang et al. reported that butachlor inhibited the incorporation of acetate into lipids of isolated leaf cells of red kidney bean (*Phaseolus vulgaris* L.). Weisshaar et al. showed a substantial inhibition of acetate incorporation into the fatty acid extracts of green alga *Scenedesmus acutus* by the short-term incorporation studies (3 hr) with [2-14C]acetate as the precursor of fatty acids. The effects of butachlor on lipid synthesis in *Chlorella*, isolated tobacco leaf cells and rice embryo cells are shown in Table 1. Lipid synthesis in *Chlorella* was sensitive to butachlor. The results showed that about one half (46%) of lipid synthesis was inhibited at 70 μM when it was incubated with butachlor for 4 hr, but no obvious inhibition was found at lower concentrations than 7 μM. In isolated tobacco leaf cells, less than 18% of lipid synthesis was inhibited at 7 μM butachlor treated, but by 70 μM concentration for 8 hr, 66% of lipid synthesis was inhibited. Similar results are found in isolated rice embryo cells. The effects of the herbicide on uptake of precursor 14C-acetate and its incorporation into lipid synthesis (4 hr) are shown in Table 2. A thirty percent difference of unincorporated acetate pool size in tobacco leaf cells was found between 70 μM of butachlor treated (4246 dpm, 71%) and untreated (2895 dpm, 41%). Although no significant difference in percentage of pool size in *Chlorella* and isolated rice embryo cells was observed between herbicide treated and untreated, the total 14C-acetate uptake at 70 μM butachlor treated (6223 and 4436 dpm for *Chlorella* and rice cells, respectively) showed only 39 and 62% of that untreated (10,390 and 7087 dpm, respectively).

Recent papers suggest that the primary site of chloroacetamides is related with lipid synthesis, especially with acetyl-CoA metabolism. CoA plays an important role in lipid metabolism and other metabolic processes that also be inhibited by chloroacetamides.

The biochemical cause of the inhibition of cell enlargement and mitosis is unknown. Inhibition of protein synthesis has been shown to result in an inhibition of mitotic entry. Duke et al. reported that a reduction in protein synthesis occurred before inhibition of growth when cucumber (*Cucumis sativus* L.) roots were treated with 24 μM propachlor. The effects of butachlor on protein synthesis in

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**Table 2** Influence of butachlor concentration on the uptake and incorporation of 14C-acetate by chlorella, tobacco leaf cell and rice embryo cell after incubation for 4 hr.

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<th>Incorporation (dpm)</th>
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<sup>a</sup> Means within columns of same plant with similar letters are not significantly different at the 5% level by Duncan's multiple range test.
Chlorella, and in isolated tobacco leaf and rice embryo cells are shown in Table 3. Incubation at 70 µM for 30 min, butachlor inhibited protein synthesis by 79, 59 and 38% for Chlorella, tobacco leaf cells, and rice embryo cells, respectively, and by 92, 95 and 85% within 8 hr. However, protein synthesis was not inhibited by butachlor at concentrations less than 7.0 µM. The effects of the herbicide on uptake of precursor ¹⁴C-leucine and incorporation into protein synthesis (4 hr) are shown in Table 4. In contrast to control, 69, 62 and 39% larger pool size of unincorporated leucine were observed in 70 µM butachlor treated Chlorella (pool size from 16 to 85%), tobacco leaf cells (24 to 86%), and rice embryo cells (31 to 70%), respectively. Total ¹⁴C-leucine uptake in 70 µM butachlor treated are found only 52, 37 and 51% of that in untreated.

Deal et al.²³ have tested the inhibition of [³H]-leucine incorporation into protein using four chloroacetamide herbicides, and reported that protein synthesis is inhibited when tested in an in vivo system, but the inhibition does not occur during the translation of mRNA into protein. However, Weisshaar & Böger²⁴ reported that chloroacetamides influenced neither the uptake of labeled lysine into the cells nor its incorporation into the DNA-associated protein fraction. The effects of butachlor on RNA synthesis in Chlorella, and

<table>
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<th>Incubation period (hr)</th>
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a) Leucine incorporated. Means within columns with similar letters are not significantly different at the 5% level by Duncan’s multiple range test.
b) Inhibition percentage means the amounts of incorporated ¹⁴C-leucine reduced from free of butachlor to different concentrations compared with free of butachlor. A minus sign indicates stimulation instead of inhibition.
isolated tobacco leaf cells and rice embryo cells are shown in Table 5. Butachlor inhibited RNA synthesis of *Chlorella* at higher concentration (70 μM), but no inhibition was observed at lower concentrations, some of results were found stimulation. Incubation under 70 μM butachlor for 8 hr, 84, 76 and 73% of RNA synthesis was inhibited in *Chlorella*, tobacco leaf cells and rice embryo cells, respectively. The effects of the herbicide on uptake of precursor 14C-uracil and its incorporation into RNA synthesis (4 hr) are shown in Table 6. Difference in percentage of pool size between 70μM butachlor treated and untreated was found varied 33, 50 and 15% in *Chlorella*, tobacco leaf cells and rice embryo cells, respectively. At that time, the total 14C-uracil uptake in 70 μM butachlor treated was 45, 73 and 47%, respectively, of that in untreated.

According to Ashton & Crafts, a primary site of action of herbicide is referable to the most obvious biochemical inhibition occurring in the lowest concentration in the shortest time. In this study, biosynthesis inhibition was not found by treating with 0.07 and 0.7 μM of butachlor in three cell types. At 7 and 70 μM butachlor, an obvious inhibition occurred in protein synthesis within 2 hr, and occurred in lipid and RNA synthesis within 4 hr. In an earlier study, we reported that 92.3 and 55.5% of the total amino acid were contained in rice seedling when cultured in a nutrient solution adding 6 and 24 ppm, respectively, of butachlor.

**ACKNOWLEDGMENTS**

This work was supported by National Science Council of the Republic of China, grant NSC 80-0409-B-002-25.

**REFERENCES**

2) E. P. Fuerst: *Weed Technol.* 1, 270 (1987)
Table 5 Influence of butachlor concentration on the incorporation of $^{14}$C-uracil in RNA synthesis of Chlorella, tobacco leaf cell and rice embryo cell during different incubation periods.

<table>
<thead>
<tr>
<th>Incubation period (hr)</th>
<th>Butachlor concentration ($\mu$M)</th>
<th>Chlorella</th>
<th>Tobacco leaf cell</th>
<th>Rice embryo cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incorp. $^a$ (dpm)</td>
<td>Inhib. $^b$ (%)</td>
<td>Incorp. $^a$ (dpm)</td>
<td>Inhib. $^b$ (%)</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>768 hi 0</td>
<td>1160 j 0</td>
</tr>
<tr>
<td></td>
<td>0.07</td>
<td>2278 fg -17</td>
<td>802 hi -4</td>
<td>1260 j -9</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>2185 g 4</td>
<td>751 hi 2</td>
<td>1189 j -3</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>2646 fg -16</td>
<td>737 hi 4</td>
<td>1158 j 0</td>
</tr>
<tr>
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<td>70</td>
<td>2086 g 8</td>
<td>589 i 23</td>
<td>936 j 19</td>
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<tr>
<td>1</td>
<td>0</td>
<td>5469 e 0</td>
<td>1744 fg 0</td>
<td>2077 h 0</td>
</tr>
<tr>
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<td>5798 e -6</td>
<td>1813 fg -4</td>
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<td>2053 h 1</td>
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<td>1603 fgh 8</td>
<td>1911 hi 8</td>
</tr>
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<td></td>
<td>70</td>
<td>3008 fg 45</td>
<td>1028 ghi 41</td>
<td>1334 ij 35</td>
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<td>0</td>
<td>9977 d 0</td>
<td>3218 e 0</td>
<td>3561 ef 0</td>
</tr>
<tr>
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<td>9546 d 4</td>
<td>3282 e -2</td>
<td>3756 e -5</td>
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<td>10,126 d -1</td>
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<td>3428 ef 4</td>
</tr>
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<td>9353 d 6</td>
<td>2798 e 13</td>
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<tr>
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<td>70</td>
<td>4011 fgc 60</td>
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<tr>
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<td>0</td>
<td>15,828 c 0</td>
<td>6076 bc 0</td>
<td>6167 c 0</td>
</tr>
<tr>
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<td>15,477 c 2</td>
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<td>6298 bc -2</td>
</tr>
<tr>
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<td>0.7</td>
<td>15,668 c 1</td>
<td>5169 d 15</td>
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<tr>
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<td>70</td>
<td>4178 fgh 74</td>
<td>1485 fghi 76</td>
<td>2348 gh 62</td>
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<td>8</td>
<td>0</td>
<td>27,856 a 0</td>
<td>8344 a 0</td>
<td>9189 a 0</td>
</tr>
<tr>
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<td>0.07</td>
<td>27,262 a 2</td>
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<td>6591 b 21</td>
<td>8937 a 2</td>
</tr>
<tr>
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<td>24,791 b 11</td>
<td>5340 cd 36</td>
<td>6856 b 25</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>4354 ef 84</td>
<td>2002 f 76</td>
<td>2513 gh 73</td>
</tr>
</tbody>
</table>

$^a$ Uracil incorporated. Means within columns with similar letters are not significantly different at the 5% level by Duncan's multiple range test.

$^b$ Inhibition percentage means the amounts of incorporated $^{14}$C-uracil reduced from free of butachlor to different concentrations compared with free of butachlor. A minus sign indicates stimulation instead of inhibition.

7) A. Liu: "Effect of Butachlor on Growth and Development of Rice and Paddy Weeds," MS Thesis, National Taiwan University, Taipei, Taiwan, 1981
Table 6  Influence of butachlor concentration on the uptake and incorporation of 14C-uracil by chlorella, tobacco leaf cell and rice embryo cell after incubation for 4 hr.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Concentration (µM)</th>
<th>Total uptake (dpm)</th>
<th>Incorporation (dpm)</th>
<th>Pool size (dpm)</th>
<th>%</th>
</tr>
</thead>
<tbody>
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<td>Chlorella</td>
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<td>15,828</td>
<td>4088</td>
<td>21 b</td>
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<tr>
<td></td>
<td>0.07</td>
<td>19,569 a</td>
<td>15,477</td>
<td>4092</td>
<td>21 b</td>
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<tr>
<td></td>
<td>0.7</td>
<td>18,702 a</td>
<td>15,668</td>
<td>3034</td>
<td>16 b</td>
</tr>
<tr>
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<td>7.0</td>
<td>18,140 b</td>
<td>15,036</td>
<td>3104</td>
<td>17 b</td>
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<td></td>
<td>70</td>
<td>9004 c</td>
<td>4178</td>
<td>4826</td>
<td>54 a</td>
</tr>
<tr>
<td>Tobacco leaf cell</td>
<td>0</td>
<td>8070 ab</td>
<td>6076</td>
<td>1994</td>
<td>25 b</td>
</tr>
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<td>6484</td>
<td>2111</td>
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<td>34 b</td>
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<td>75 a</td>
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<td>Rice embryo cell</td>
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<td>6167</td>
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<td>2348</td>
<td>1397</td>
<td>37 a</td>
</tr>
</tbody>
</table>

* Means within columns of same plant with similar letters are not significantly different at the 5% level by Duncan’s multiple range test.