Characterization of Methotrexate Transport and Its Drug Interactions with Human Organic Anion Transporters

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ABSTRACT
Life-threatening drug interactions are known to occur between methotrexate and nonsteroidal anti-inflammatory drugs (NSAIDs), probenecid, and penicillin G. The purpose of this study was to characterize methotrexate transport, as well as to determine the site and the mechanism of drug interactions in the proximal tubule. Mouse proximal tubule cells stably expressing basolateral human organic anion transporters (hOAT1 and hOAT3) and apical hOAT (hOAT4) were established. The \( K_{m} \) values for hOAT1-, hOAT3-, and hOAT4-mediated methotrexate uptake were 553.8 \( \mu \)M, 21.1 \( \mu \)M, and 17.8 \( \mu \)M, respectively. NSAIDs (salicylate, ibuprofen, ketoprofen, phenylbutazone, piroxicam, and indomethacin), probenecid, and penicillin G dose dependently inhibited methotrexate uptake mediated by hOAT1, hOAT3, and hOAT4. Kinetic analysis of inhibitory effects of these drugs on hOAT3-mediated methotrexate uptake revealed that these inhibitions were competitive. The \( K_{i} \) values for the effects of salicylate, phenylbutazone, indomethacin, and probenecid on hOAT3-mediated methotrexate uptake were comparable with therapeutically relevant plasma concentrations of unbound drugs. In addition, the presence of human serum albumin, the \( K_{i} \) values were comparable with therapeutically relevant total plasma concentrations of drugs. In conclusion, these results suggest that methotrexate is taken up via hOAT3 and hOAT1 at the basolateral side of the proximal tubule and effluxed or taken up at the apical side via hOAT4. In addition, hOAT1, hOAT3, and hOAT4 are the sites of drug interactions between methotrexate and NSAIDs, probenecid, and penicillin G. Furthermore, it was predicted that hOAT3 is the site of drug interactions between methotrexate and salicylate, phenylbutazone, indomethacin, and probenecid in vivo.

Methotrexate is widely used at high dosages in the treatment of malignancies, whereas it is used at low dosages in rheumatoid arthritis. Methotrexate is eliminated almost entirely in an unchanged form in urine, which involves glomerular filtration and active tubular secretion (Shen and Azarnoff, 1978). Therefore, renal insufficiency or drug interactions, which reduce the clearance of methotrexate, are potentially toxic events.

Interactions between methotrexate and drugs including nonsteroidal anti-inflammatory drugs (NSAIDs), probenecid, and penicillin G have been reported by several groups of investigators (Ellison and Servi, 1985; Thyss et al., 1986; Basen et al., 1991; Frenia and Long, 1992). The interactions may have been caused by protein binding displacement, inhibitory effects on the renal secretion of methotrexate, and a decline in glomerular filtration as a result of inhibition of prostaglandin synthesis (Tracy et al., 1992; Kremer and Hamilton, 1995). Among these possible causes, the renal tubular secretion of methotrexate has been thought to be a major site for drug interaction (Frenia and Long, 1992).

Two different types of human multispecific OATs (hOAT1 and hOAT3) were recently isolated (Reid et al., 1998; Hosoyama, et al., 1999; Cha et al., 2001). In the kidney, hOAT1 and hOAT3 are localized at the basolateral membrane of the proximal tubule (Hosoyama et al., 1999; Cha et al., 2001). Since hOAT1 and hOAT3 mediate the transport of various drugs, endogenous substances, and xenobiotics, these transporters are considered to be responsible for the basolateral...
uptake of organic anions in renal epithelial cells. On the other hand, OAT-K1 (Saito et al., 1996; Masuda et al., 1999b), OAT-K2 (Masuda et al., 1999a), hOAT4 (Cha et al., 2000), organic anion-transporting peptide 1 ( oatp1) (Jacque-min et al., 1994), oatp3 (Abe et al., 1998), multidrug-resis-
tance protein 2 (MRP2) (Leier et al., 2000), and human type I sodium-dependent inorganic phosphate transporter (NPT1) (Uchino et al., 2000) were isolated and identified as trans-
porters of anionic drugs and substances at the apical mem-
brane of the proximal tubule.

The purpose of this study was to characterize methotrexate transport in the proximal tubule, as well as to determine the site and mechanism of interactions between methotrexate and NSAIDs, probenecid, and penicillin G using the second portion of the proximal tubule (S2) cells stably expressing hOAT1, hOAT3, and hOAT4 (S2 hOAT1, S2 hOAT3, and S2 hOAT4, respectively).

Experimental Procedures

Materials. [3H]Methotrexate (547.6 GBq/mmol) was purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckingham-
shire, UK). Methotrexate, various NSAIDs, probenecid, penicillin G, transferin, and human serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). Other materials used included fetal bovine serum, trypsin and geneticin from Invitrogen (Carlsbad, CA), recombinant epidermal growth factor from Wakunaga (Hiroshima, Japan), insulin from Shimizu (Shizuoka, Japan), RITC 80–7 culture medium from Iwaki Co. (Tokyo, Japan), and TTX-50 from Promega (Madison, WI).

Cell Culture and Establishment of S2 hOAT1, S2 hOAT3, and S2 hOAT4. S2 cells, derived from transgenic mice harboring temperature-sensitive simian virus 40 large T-antigen gene, were established as described previously by us (Takeda et al., 1999). S2 is the segment of the proximal tubule in which hOAT1, hOAT3, and hOAT4 were localized (Hosoyamada et al., 1999; Cha et al., 2001; Babu et al., 2002). The full-length cDNAs of hOAT1, hOAT3, and hOAT4 were subcloned into pcDNA 3.1 (Invitrogen, Carlsbad, CA), a mammalian expression vector. S2 hOAT1, S2 hOAT3, and S2 hOAT4 were obtained by transfecting S2 cells with pcDNA3.1-hOAT1, pcDNA3.1-hOAT3, and pcDNA3.1-hOAT4 coupled with pSV2neo, a neomycin-resistant gene, using TITX-50 according to the manufactur-
er’s instructions. S2 cells transfected with pcDNA3.1 lacking an insert, and pSV2neo were designated as S2-pcDNA 3.1 and used as a control (mock). These cells were grown in a humidified incubator at 33°C and under 5% CO2 using RITC 80–7 medium containing 5% fetal bovine serum, 10 μg/ml transferrin, 0.08 μM insulin, 10 ng/ml recombinant epidermal growth factor, and 400 μg/ml Geneticin. The cells were subcultured in a medium containing 0.05% trypsin-EDTA solution (containing 137 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 4 mM NaHCO3, 0.5 mM EDTA, and 5 mM Hepes, pH 7.2) and used for 10 to ≈35 passages. Clonal cells were isolated using a cloning cylin-
der and screened by determining the uptake of the optimal substrate

Uptake Experiments. Uptake experiments were performed as previously described (Takeda et al., 1999). As described above, the cells were cultured at 33°C, which is suitable for cell growth for cells encoding temperature-sensitive simian virus 40 large T-antigen gene (Takeda et al., 1999). In contrast, uptake experiments were performed at 37°C, which is commonly used for evaluating transporter activities. There was no difference in the amount of uptake between 33°C and 37°C (data not shown). Based on these results, the cells were cultured at 33°C, whereas uptake experiments were performed at 37°C. The S2 cells were seeded in 24-well tissue culture plates at a cell density of 1 × 105 cells/well. After the cells were cultured for 2 days, the cells were washed three times with Dulbecco’s modified phosphate-buffered saline (D-PBS) solution (containing 137 mM NaCl, 3 mM KCl, 0.85% NaH2PO4, 1 mM KH2PO4, 1 mM CaCl2, and 0.5 mM MgCl2, pH 7.4), and then preincubated in the same solution for 10 min in a water bath at 37°C. The cells were then incubated in D-PBS containing [3H]methotrexate at various concentrations as indicated in each experiment. The uptake was stopped by the addi-
tion of ice-cold D-PBS, and the cells were washed three times with the same solution. The cells in each well were lysed with 0.5 ml of 0.1 N sodium hydroxide and 2.5 ml of Aquasol-2, and radioactivity was determined using a β-scintillation counter (Aloka, Tokyo, Japan; LSC-3100).

Inhibition Study. To evaluate the inhibitory effects of various NSAIDs, probenecid, and penicillin G on methotrexate uptake mediated by hOAT1, hOAT3, and hOAT4, S2 hOAT1, S2 hOAT3, and S2 hOAT4 were incubated in a solution containing [3H]methotrexate in the absence or presence of various drugs at 37°C. Considering the Kᵢ values for hOAT1-, hOAT3-, and hOAT4-mediated methotrexate uptake, hOAT1-, hOAT3-, and hOAT4-mediated methotrexate uptake was estimated using a methotrexate concentration of 1 μM (hOAT1) or 100 nM (hOAT3 and hOAT4). In addition, based on the results of time course experiments (Fig. 1), hOAT3-mediated methotrexate uptake was evaluated during the 2-min incubation. On the other hand, since the specific methotrexate uptake activity, represented as the amount of hOAT1- and hOAT4-mediated methotrexate uptake subtracted by that by mock, was about two times larger during the 15-min incubation than that during the 2-min incubation, we chose 15 min as the incubation time for the inhibition experiments. Sali-
ylate, probenecid, and penicillin G were dissolved in distilled water.

Kinetic Analysis. After preincubation as described above, S2 hOAT3 was incubated in D-PBS containing [3H]methotrexate at different concentrations in the absence or presence of various drugs for 2 min. For the kinetic analysis of inhibitory effects, double recipro-
cal plot analyses were performed based on methotrexate under each condition (APIwattanakul et al., 1999). When the inhibition was competitive, the Kᵢ values were calculated based on the following equation,

\[ Kᵢ = \frac{[I]}{[Kᵢ \text{ of methotrexate with inhibitor}] - 1} \]

where \( Kᵢ \) is the inhibition constant.

Statistical Analysis. Data are expressed as means ± S.E. Statis-
tical differences were determined using one-way ANOVA with Dunnett’s post hoc test. Differences were considered significant at \( P < 0.05 \).

Results

Methotrexate Uptake. To evaluate the time-dependent uptake of methotrexate in S2 hOAT1, S2 hOAT3, and S2 hOAT4, S2 cell monolayers were incubated in a solution
the two separate experiments.

Trexl (hOAT3 and hOAT4) for various periods at 37°C. The values for the specific uptake in S2 hOAT1, S2 hOAT3, and S2 hOAT4 (open circles) were obtained by subtracting that in mock (closed circles) from those in S2 hOAT1, S2 hOAT3, and S2 hOAT4 (open circles). Each value represents the mean ± S.E. of four determinations from one typical experiment of the two separate experiments.

Inhibitory Effects on Methotrexate Uptake. We examined the effects of various drugs on methotrexate uptake mediated by hOAT1, hOAT3, and hOAT4. The drugs used were NSAIDs (salicylate, ibuprofen, ketoprofen, phenylbutazone, piroxicam, and indomethacin), probenecid, and penicillin G. These drugs inhibited methotrexate uptake mediated by hOAT1, hOAT3, and hOAT4 in a dose-dependent manner, whereas penicillin G did not inhibit hOAT1-mediated methotrexate uptake and salicylate did not inhibit hOAT4-mediated methotrexate uptake (data not shown). The effects of these NSAIDs on methotrexate uptake by hOAT1, hOAT3, and hOAT4 are listed in Table 1.

Kinetic Analysis of Inhibition. To further elucidate the inhibitory effects of NSAIDs, probenecid, and penicillin G on hOAT3-mediated methotrexate uptake, we have examined the inhibitory effects of these drugs at different concentrations of methotrexate. Typical results are shown in Fig. 3. Analysis of a Lineweaver-Burk plot of the effects of salicylate (Fig. 3A), phenylbutazone (Fig. 3B), indomethacin (Fig. 3C), and probenecid (Fig. 3D) on the hOAT3-mediated methotrexate uptake demonstrated that these drugs inhibited the methotrexate uptake in a competitive manner. The inhibitory effects of ibuprofen, ketoprofen, piroxicam, and penicillin G were also competitive (data not shown). The K_i values of methotrexate uptake by hOAT1, hOAT3, and hOAT4 were measured in a competitive manner. The inhibitory effects of these drugs at different concentrations of methotrexate were studied. The results suggested that hOAT1, hOAT3, and hOAT4 mediated transport of methotrexate.

Fig. 2. Eadie-Hofstee plot analysis of methotrexate uptake in S2 hOAT1 (A), S2 hOAT3 (B), and S2 hOAT4 (C). S2 hOAT1, S2 hOAT3, and S2 hOAT4 were incubated with solution containing 1 μM [3H]methotrexate in the absence or presence of various NSAID drugs. The concentrations of NSAIDs used were 1 μM except 0.5 μM for indomethacin. The values for the uptake in mock were subtracted from those in S2 hOAT1, S2 hOAT3, and S2 hOAT4. Each value represents the mean ± S.E. of four determinations from one typical experiment of the two separate experiments.

TABLE 1

<table>
<thead>
<tr>
<th>Drugs</th>
<th>hOAT1</th>
<th>hOAT3</th>
<th>hOAT4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicylate</td>
<td>44.7 ± 7.95***</td>
<td>41.5 ± 2.90*</td>
<td>94.8 ± 8.53</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>47.0 ± 3.05*</td>
<td>64.0 ± 3.30**</td>
<td>67.8 ± 5.23**</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>53.4 ± 5.43**</td>
<td>45.2 ± 3.45*</td>
<td>81.0 ± 1.78***</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>30.0 ± 1.13*</td>
<td>31.3 ± 2.43*</td>
<td>55.7 ± 7.20*</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>40.9 ± 10.7*</td>
<td>56.2 ± 3.82*</td>
<td>62.0 ± 8.34***</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>60.6 ± 7.28*</td>
<td>38.5 ± 3.11*</td>
<td>60.9 ± 5.74**</td>
</tr>
<tr>
<td>Probenecid</td>
<td>53.1 ± 4.29*</td>
<td>30.2 ± 4.32*</td>
<td>81.6 ± 6.26***</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>102.3 ± 6.18</td>
<td>27.8 ± 2.56*</td>
<td>79.7 ± 4.88***</td>
</tr>
</tbody>
</table>

* P < 0.001, ** P < 0.01, and *** P < 0.05 versus control.
concentrations of [3H]methotrexate in the presence or absence of various
eral side of the S2 segment of the proximal tubule (Hosoy-
substrate specificity and localization: hOAT1 at the basolat-
characteristics exist between hOAT1 and hOAT3, such as
in the basolateral membrane of the proximal tubule (Hosoy-
as multispecific OATs, and were found to mediate active
Fig. 3. Kinetic analysis of inhibition by various drugs on methotrexate
uptake in S1 hOAT3. Methotrexate uptake was measured at various
carrier-mediated transport of methotrexate in the basolat-
for each drug tested are shown in Table 2. In addition, we
for salicylate, phenylbutazone, indomethacin, and probenecid on
on hOAT3-mediated methotrexate uptake in the presence of
Discussion
hOAT1 and hOAT3 were recently cloned and characterized
as multispecific OATs, and were found to mediate active transport of organic anions from the interstitium to the cells in the basolateral membrane of the proximal tubule. Some differences in characteristics exist between hOAT1 and hOAT3, such as substrate specificity and localization: hOAT1 at the basolateral side of the S2 segment of the proximal tubule versus hOAT3 at the first, second, and third segments (S1, S2, and S3) of the proximal tubule. In addition, hOAT1, but not hOAT3, exhibits transport properties as an exchanger. On the other hand, hOAT4 is localized to the apical membrane of the proximal tubule (Babu et al., 2002), and hOAT4 exhibits a relatively narrow substrate specificity compared with hOAT1 and hOAT3 (Cha et al., 2001).

Carrier-mediated transport of methotrexate in the basolat-
eral side of the proximal tubule was suggested based on the results of the experiments using renal slices (Nierenberg, 1983; Williams et al., 1984) or isolated proximal tubule (Besseghir et al., 1989). The current results suggest that hOAT3 and hOAT1 mediate the transport of methotrexate in the basolateral side of the proximal tubule. Human OAT cloned by Lu was designated as human kidney p-aminobenzoate transporter (hPAHT) (Lu et al., 1999), and there was 97.2% amino acid sequence homology between hPAHT and hOAT1 (data not shown). In contrast to hOAT1, hPAHT exhibited no significant methotrexate uptake activity. The discrepancy in methotrexate uptake activity between hOAT1 and hPAHT may be due to this small difference in amino acid sequence or the experimental conditions. To clarify this, a site-directed mutagenesis study should be performed. The current results were also consistent with the previous results that rat OAT1 mediates methotrexate uptake (Uwai et al., 2000). In addition, hOAT2, which was recently identified to be localized to the basolateral side of the proximal tubule, showed no significant uptake activity of methotrexate (M. Takeda, unpublished observation). This is in contrast to the recent report that cells expressing hOAT2 showed uptake of methotrexate (Sun et al., 2001). The reason for the discrepancy remains unknown, and further studies should be performed. It is also possible that unidentified OATs other than hOAT3 and hOAT1 are also involved in methotrexate transport in the basolateral membrane of the proximal tubule.

In addition to hOAT4, human transporters mediating the transport of organic anions in the apical membrane of the proximal tubule have been cloned, including MRPs (Leier et al., 2000) and NPT1 (Uchino et al., 2000). Moreover, human MRP2 was shown to mediate the efflux of methotrexate using stable cells (Hooijberg et al., 1999). Thus, the relative contribution of hOAT4 and MRPs to apical methotrexate transport should be clarified. In addition to human transporters, rodent transporters mediating the apical transport of organic anions have been cloned, including OAT-K1 (Saito et al., 1996; Masuda et al., 1999a), OAT-K2 (Masuda et al., 1999b), oatp1 (Jacquemin et al., 1994), and oatp3 (Abe et al., 1998). Among them, OAT-K1 and OAT-K2 were reported to mediate bidirectional methotrexate transport (Saito et al., 1996; Masuda et al., 1999a,b), and the Km value for OAT-K1-mediated methotrexate transport was determined to be 1.0 μM (Masuda et al., 1997). Thus, the cloning of human homologs of these rodent transporters and the functional analysis for the role of these transporters in apical methotrexate transport should be performed.

Since various drugs tested in this study dose dependently inhibited hOAT1-, hOAT3-, and hOAT4-mediated methotrexate uptake, it was suggested that hOAT1, hOAT3, and hOAT4 are sites of drug interactions between methotrexate and these drugs. Among these interactions, it appears that those between methotrexate and salicylate, phenylbutazone, indomethacin, or probenecid at hOAT3 may have clinical significance, as follows. The total and unbound plasma concentrations of various drugs are as listed in the Table 2. Therapeutically relevant concentrations of unbound drugs in the plasma are thought to be within 5-fold of the maximum steady-state concentrations of unbound drugs in the plasma (Zhang et al., 2000). Comparing the Km values with therapeutically relevant concentrations of unbound drugs in the plasma, those for salicylate, phenylbutazone, indomethacin,
The $K_i$ values of various drugs that inhibit methotrexate uptake in S2 hOAT3 are presented in Table 2. hOAT3 was incubated with solution containing various concentrations of $[3H]$methotrexate in the absence or presence of various drugs. Experiments were performed in the absence or presence of 5% human serum albumin (HSA). The values for the uptake in mock were subtracted from those in S2 hOAT3. The unbound plasma concentrations were calculated from the total plasma concentrations and unbound fractions of drugs.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>$K_i$ HSA(−)</th>
<th>$K_i$ HSA(+)</th>
<th>Concentration of Drugs</th>
<th>Total Plasma Concentration</th>
<th>Unbound Fraction</th>
<th>Unbound Plasma Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicylate</td>
<td>1020</td>
<td>2440</td>
<td>2000</td>
<td>2000</td>
<td>2200^*</td>
<td>19.6^*</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>1170</td>
<td>1160</td>
<td>2000</td>
<td>2000</td>
<td>48.5^a</td>
<td>1.00&lt;^&lt;a</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>34.7</td>
<td>1200</td>
<td>350</td>
<td>50</td>
<td>1.20^b</td>
<td>0.80^b</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>5.95</td>
<td>29.8</td>
<td>10</td>
<td>50</td>
<td>84.0^c</td>
<td>10.0^c</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>97.6</td>
<td>97.6</td>
<td>110</td>
<td>50</td>
<td>170^e</td>
<td>11.0^e</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>100</td>
<td>100</td>
<td>160</td>
<td>50</td>
<td>9.60^f</td>
<td>49.1^f</td>
</tr>
</tbody>
</table>


and probenecid were comparable. In addition, the $K_i$ values obtained in the presence of 5% human serum albumin were comparable with total plasma concentrations of salicylate, phenylbutazone, indomethacin, or probenecid. Based on these, it was predicted that these drugs could inhibit the hOAT3-mediated methotrexate uptake in vivo, leading to the increased plasma concentration of methotrexate.

Uwai et al. (2000) have already demonstrated that the IC$_{50}$ values of salicylate, ketoprofen, and indomethacin for rat OAT1-mediated methotrexate uptake were 1410, 0.5, and 2.7 µM, respectively. However, we found that the IC$_{50}$ values of salicylate, ketoprofen, and indomethacin for hOAT1-mediated methotrexate uptake were >2000, 1200, and >2000 µM, respectively (data not shown). This discrepancy in findings may be due to the species difference, i.e., between rats and humans. Compared with rat OAT1, the amino acid sequence of hOAT1 exhibited 86.0% homology (Sekine et al., 1997). On the other hand, the IC$_{50}$ values of ibuprofen, ketoprofen, piroxicam, indomethacin, and probenecid for adefovir uptake in Chinese hamster ovary cells stably expressing hOAT1 were 8.0, 1.3, 20.5, 3.0, and 7.4 µM, respectively (Mulato et al., 2000). The results were similar to those for rat OAT1-mediated methotrexate uptake (Uwai et al., 2000), but much lower than those for hOAT1-mediated methotrexate uptake. The discrepancy may be due to the fact that the $K_m$ value for hOAT1-mediated adefovir uptake was 23.8 µM (Ho et al., 2000), whereas that for hOAT1-mediated methotrexate uptake was 553.8 µM.

At present, the mechanism for the interaction between methotrexate and ibuprofen, ketoprofen, or piroxicam, or penicillin G remains unclear. It is possible that other transporters mediate methotrexate transport and are responsible for drug interactions. Since protein-binding displacement of methotrexate by NSAIDs except salicylate is generally thought to account for more than a small transient increase in free methotrexate concentration, protein binding changes would probably not be of major clinical significance (Taylor and Halprin, 1977). In addition, since prostaglandin synthesis is stimulated to maintain the glomerular filtration rate in the setting of prerenal volume contraction (Ciabattoni et al., 1984; Dunn, 1984), NSAIDs may decrease glomerular filtration of methotrexate as a result of inhibition of prostaglandin synthesis (Kremer and Hamilton, 1995). However, patients who do not have a condition that predisposes them to activation of renal prosta glandins would not have a decrease in renal function following treatment with NSAIDs (Kremer and Hamilton, 1995).

In conclusion, these results suggest that methotrexate is taken up in the basolateral membrane by hOAT3 and hOAT1, and taken up or effluxed in the apical membrane of the proximal tubule via hOAT4. In addition, hOAT1, hOAT3, and hOAT4 were sites of drug interactions between methotrexate and NSAIDs, probenecid, and penicillin G. Furthermore, it was predicted that hOAT3 is a site of drug interactions between methotrexate and salicylate, phenylbutazone, indomethacin, or probenecid in vivo. Stable cells established in this study provide a good opportunity for evaluating drug interaction of methotrexate and newly developed drugs, particularly NSAIDs, in the preclinical stage.

References


Holland et al. (1960), ^f Peterson et al. (1977).