Prostaglandin Endoperoxide Synthetase-Dependent Cooxidation of Acetaminophen to Intermediates which Covalently Bind in Vitro to Rabbit Renal Medullary Microsomes

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ABSTRACT

The metabolism of acetaminophen during prostaglandin biosynthesis was studied in vitro. [3H]Acetaminophen was rapidly metabolized by ram seminal vesicle microsomes to an intermediate(s) which covalently binds to microsomal protein. Arachidonic acid, a substrate for the fatty acid cyclooxygenase component of prostaglandin endoperoxide synthetase (PES), was required to support binding. The cyclooxygenase inhibitor indomethacin inhibited binding. Cumene hydroperoxide, a substrate for the hydroperoxidase component of PES, supported covalent binding which was not inhibitable by indomethacin.

Acetaminophen also stimulated oxygen uptake during prostaglandin biosynthesis in a dose-dependent manner. By using a purified PES preparation with bovine serum albumin as the acceptor protein, similar results were obtained. Binding was dependent upon the presence of PES and arachidonic acid and was inhibited by indomethacin. Experiments were then carried out by using microsomes prepared from the rabbit kidney medulla, a possible target organ of acetaminophen-induced toxicity. Arachidonic acid and cumene hydroperoxide supported covalent binding, whereas indomethacin inhibited binding supported by the former but not the latter. Reduced glutathione prevented covalent binding to protein when added to the incubation mixture and NADPH did not support binding. These results suggest that the cooxidation of acetaminophen during prostaglandin biosynthesis may play a role in the renal metabolism and possible nephrotoxicity of this compound.

Acetaminophen (N-acetyl-p-aminophenol, paracetamol) is a widely used analgesic and antipyretic drug, proven extremely safe in therapeutic doses, but capable of inducing hepatic necrosis after overdosage in humans (Boyd and Bereczky, 1966; Prescott et al., 1971). More recently, renal tubular necrosis has been observed in conjunction with hepatotoxicity in rodents and humans (McMurtry et al., 1978; Boyer and Rouff, 1971). There is also evidence of renal damage occurring without significant hepatic damage (Mitchell et al., 1977; Maclean et al., 1968; Prescott et al., 1971; Kincaid-Smith, 1980). The currently accepted biochemical mechanism for acetaminophen-induced hepatic toxicity requires the microsomal cytochrome P-450-dependent mixed-function oxidase system to metabolize acetaminophen to an electrophilic intermediate, which covalently binds to tissue protein and/or macromolecules, resulting in cellular necrosis (Hinson, 1980).

A variety of xenobiotics may undergo cooxidation to reactive intermediates during prostaglandin biosynthesis in RSV (Marnett et al., 1979; Sivarajah et al., 1979), human lung and other mammalian microsomal systems (Sivarajah et al., 1981). Davis and co-workers (1981) have shown the prostaglandin-mediated metabolism of several compounds in rabbit renal medulla tissue, a rich source of PES, but lacking in mixed-function oxidase activity. Other workers, by using antibodies to PES, report that high concentrations of this enzyme exist in areas of the kidney where acetaminophen toxicity is observed (Smith and Wilkin, 1977). In addition, the chemical nature of acetaminophen suggests susceptibility to one electron oxidation (De Vries, 1981). This led us to investigate the possibility of PES-dependent formation of reactive acetaminophen intermediates which covalently bind to macromolecules. While this work was in progress, Moldeus and Rahimtula (1980) reported the formation of an acetaminophen-glutathione conjugate during prostaglandin biosynthesis in RSV microsomes.

Methods

Chemicals. [G-3H]Acetaminophen (9.3 mCi/mmol) was purchased from New England Nuclear (Boston, MA) and was purified by the methods of Mudge et al. (1978). Unlabeled acetaminophen was pur-
charged from Sigma Chemical Company (St. Louis, MO). Arachidonic acid (99% pure) was purchased from Nu Chek Prep, Inc. (Elysian, MN). Cumene hydroperoxide was purchased from Pfaltz and Bauer, Inc. (Stamford, CT). Liquid phenol (90%) was purchased from Fisher Scientific Company (Fairlawn, NJ). Hematin, NADPH, indomethacin and reduced glutathione were purchased from Sigma. Aquasol scintillation fluid was purchased from New England Nuclear.

**RSV microsomes.** RSVs were obtained from a slaughter house and stored at −60°C until needed. Microsomes were prepared as previously described (Parkes and Eling, 1974), except that initial washings were carried out in 1.15% KCl containing 20 mM Tris-KCl buffer, pH 7.4, and microsomes were resuspended in 0.1 M phosphate buffer, pH 7.8, before storage. Protein determination was made by the method of Lowry et al. (1951). Incubation mixtures for covalent binding studies consisted of 0.05 M phosphate buffer, pH 7.8, 2 mg of RSV microsomal protein, 60 μM [3H]acetaminophen (4.5 × 10^6 dpm) and water to make 2 ml. Incubations were carried out at 37°C for 10 min before incubation. Reactions were carried out at 37°C for 5 min in a Dubnoff shaking incubator and were terminated by the addition of 8 ml of ethyl acetate with immediate vortex. The organic layer was then removed after centrifugation at 1500 rpm for 10 min in a Sorvall GLC-1 centrifuge, and protein was precipitated by the addition of the 2 ml of 10% trichloroacetic acid. After two additional trichloroacetic acid washes, the protein was repeatedly extracted with 80% methanol until no radioactivity above background could be detected in the extract (six to eight extractions). Any remaining radioactivity was then considered to be covalently bound to the protein (Jollow et al., 1973). The protein was digested in 1 N NaOH at 55°C for 24 hr. An aliquot was counted by using standard liquid scintillation techniques and protein concentration was determined. Covalent binding was expressed as nanomoles of acetaminophen bound per milligram of RSV protein.

Oxygen uptake studies were carried out with a Clark oxygen electrode maintained at 37°C. Incubation mixtures were identical with that described above except that unlabeled acetaminophen was added in various concentrations as indicated. Oxygen uptake during prostaglandin biosynthesis was recorded on a Hewlett-Packard 7132-A chart recorder and slopes were compared with controls to determine percentage increase in O_2 uptake.

**Purified PES.** PES, purified to homogeneity, was obtained from Dr. William Pagels (Wayne State University, Detroit, MI). This procedure is in manuscript and will be published shortly. Complete incubation mixtures consisted of 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.8; 2 mg of crystallized BSA (Armour Pharmaceutical Company, Chicago, IL); 10 μl of purified PES; 500 μM phenol; 1 μM hematin; 100 μM [3H]acetaminophen (5 × 10^6 dpm); 200 μM arachidonic acid; and water to make 2 ml. Test mixtures consisted of the above with the addition of 200 μM indomethacin or deletions as indicated. Incubations were carried out at 37°C for 10 min and terminated with ethyl acetate. Protein precipitation, extraction and determination of covalent binding were carried out as previously described for RSV experiments. Covalent binding was expressed as nanomoles of acetaminophen bound per milligram of protein.

**Rabbit renal medullary microsomes.** Adult male New Zealand White rabbits (Dutchland Laboratories, Denver, PA) weighing 2.0 to 2.5 kg were sacrificed with i.v. injections of sodium pentobarbital. Kidneys were quickly removed and the medulla was carefully dissected away from the cortex. Medullary tissue was placed in ice-cold 1.15% KCl buffer, pH 7.4, and microsomes were prepared as described earlier for RSV microsomes. Time course and concentration-dependent studies consisted of the following incubation mixture: 0.05 M phosphate buffer, pH 7.8; 2 mg of medullary microsomes; 100 μM [3H]acetaminophen (4.5 × 10^6 dpm) (10 μM-2mM for concentration-dependence studies) 200 μM arachidonic acid; and water to make 2 ml. Incubations were carried out at 37°C for 10 min (10 sec-20 min for time course) and terminated with ethyl acetate as described earlier. Boiled blanks contained protein which had been boiled for 15 min before incubation.

**Results**

**RSV microsomes.** Investigation of acetaminophen metabolism by PES is complicated by the fact that acetaminophen is an inhibitor of PES (Aiken, 1974). We first examined the effect of acetaminophen on PES by measuring the incorporation of oxygen in arachidonic acid metabolism using RSV microsomes as the source of PES. As seen in figure 1, acetaminophen stimulates oxygen uptake and hence PES. At a concentration of approximately 200 μM, a 50% increase in O_2 uptake was observed. Beyond a concentration of 3 mM, inhibition of PES was observed. In all further RSV microsome experiments, an acetaminophen concentration of 60 μM was used. The PES system in RSV microsomes supported the conversion of acetaminophen to covalently bound material (table 1). Indomethacin, an inhibitor of the cyclooxygenase component of PES, significantly reduced the covalent binding. Arachidonic acid was required for covalent binding and little binding was observed by using boiled microsomes. The addition of cumene

![Graph](https://via.placeholder.com/150)

**Table 1**

<table>
<thead>
<tr>
<th>Acetaminophen Bound/mg Protein</th>
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</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>3.424 ± 0.234</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>1.309 ± 0.152</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>1.438 ± 0.155</td>
</tr>
<tr>
<td>Complete system</td>
<td>3.014 ± 0.326</td>
</tr>
<tr>
<td>Cumene hydroperoxide</td>
<td>1.308 ± 0.152</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>2.815 ± 0.117</td>
</tr>
</tbody>
</table>

* Complete system contains: 0.05 M phosphate buffer, pH 7.8; 2 mg RSV microsomal protein; 60 μM [3H]acetaminophen; 100 μM arachidonic acid; and water to make 2 ml. Indomethacin = 200 μM.

* Complete system contains: same as above with 100 μM cumene hydroperoxide instead of arachidonic acid. Indomethacin = 200 μM.
Hydroperoxide also supported the oxidation of acetaminophen as measured by covalent binding to protein. Indomethacin, which inhibits the formation of the hydroperoxide prostaglandin G2 from arachidonic acid but not the peroxidase component of PES, did not inhibit the covalent binding of acetaminophen to protein in the presence of cumene hydroperoxide.

Purified PES. Purified PES and BSA as the acceptor protein catalyzed the covalent binding of acetaminophen to the protein. Significantly less covalent binding was observed in incubation mixtures containing no enzyme. The deletion of arachidonic acid or addition of indomethacin greatly reduced covalent binding. The addition of phenol to the incubation mixture supported the maximal level of covalent binding (table 2).

**Rabbit renal medullary microsomes.** [3H]Acetaminophen was incubated with rabbit renal medullary microsomes at different substrate concentrations and for varying lengths of time. The reaction was approximately linear (fig. 2) up to 5 min and was saturated at 10 min; this time point (10 min) was chosen for all further experiments. Examination of concentration dependence showed saturation occurring at approximately 100 μM (fig. 3). K_m and V_max values for acetaminophen covalent binding to medullary microsomes were estimated by a Lineweaver-Burk double reciprocal plot. As seen in figure 4, a K_m of approximately 40 μM and a V_max of 0.91 nmol/mg/min were obtained.

The addition of arachidonic acid to incubation mixtures was found to support the covalent binding of 100 μM [3H]acetaminophen to rabbit renal medullary microsomal protein (table 3).

**TABLE 2**

<table>
<thead>
<tr>
<th>Purified PES-dependent covalent binding of acetaminophen to BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>All values are mean ± S.D., N = 3.</td>
</tr>
<tr>
<td>Acetaminophen Bound/mg Protein (nmol)</td>
</tr>
<tr>
<td>Complete system*</td>
</tr>
<tr>
<td>12.10 ± 1.09</td>
</tr>
<tr>
<td>- Purified PES</td>
</tr>
<tr>
<td>2.35 ± 0.51</td>
</tr>
<tr>
<td>- Phenol</td>
</tr>
<tr>
<td>6.25 ± 0.25</td>
</tr>
<tr>
<td>- Arachidonic acid</td>
</tr>
<tr>
<td>2.66 ± 0.16</td>
</tr>
<tr>
<td>+ Indomethacin</td>
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<tr>
<td>2.49 ± 0.82</td>
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</tbody>
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* Complete system contains: 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.8; 10 μl of purified PES; 500 μM phenol; 1 μM hematin; 100 μM [3H]acetaminophen; 2 mg of BSA; 200 μM arachidonic acid; and water to make 2 ml. Indomethacin = 200 μM.

**Fig. 3.** Relationship between acetaminophen concentration and covalent binding to rabbit renal medullary microsomes. Bars represent S.D., N = 3.

**Fig. 4.** Lineweaver-Burk plot of acetaminophen covalent binding to rabbit renal medullary microsomes. I/S, reciprocal substrate concentration; I/V, reciprocal velocity.

**TABLE 3**

<table>
<thead>
<tr>
<th>Arachidonic acid-dependent covalent binding of acetaminophen to rabbit renal medullary microsomes</th>
</tr>
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<tbody>
<tr>
<td>All values are mean ± S.D., N = 3, boiled blank values subtracted.</td>
</tr>
<tr>
<td>Acetaminophen Bound/mg Protein (nmol)</td>
</tr>
<tr>
<td>Complete system*</td>
</tr>
<tr>
<td>1.028 ± 0.045</td>
</tr>
<tr>
<td>- Arachidonic acid</td>
</tr>
<tr>
<td>0.037 ± 0.023</td>
</tr>
<tr>
<td>+ Indomethacin</td>
</tr>
<tr>
<td>0.308 ± 0.065</td>
</tr>
<tr>
<td>- Arachidonic acid + NADPH</td>
</tr>
<tr>
<td>0.005 ± 0.004</td>
</tr>
<tr>
<td>+ Glutathione</td>
</tr>
<tr>
<td>0.016 ± 0.014</td>
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</table>

* Complete system contains: 0.05 M phosphate buffer, pH 7.8; 2 mg of rabbit kidney medullary microsomes; 200 μM arachidonic acid; 100 μM [3H]acetaminophen; and water to make 2 ml. Indomethacin = 200 μM; NADPH = 1 mM; and glutathione = 1 mM.

Very little binding occurred in the absence of a PES substrate. The addition of indomethacin, an inhibitor of the cyclooxygenase component of PES, inhibited binding by approximately 70%. NADPH did not support binding in the absence of arachidonic acid. Reduced glutathione virtually eliminated covalent binding when added to the incubation mixture. When added after 10 min, glutathione had no effect on binding. Cumene hydroperoxide, a substrate for the hydroperoxidase component of PES, supported covalent binding of acetaminophen...
phen to microsomal protein (table 4). The addition of indo-
methacin to this reaction mixture had little inhibitory effect.

It should be noted that in all covalent binding experiments
there was a relatively high level of nonspecific binding of
acetaminophen to microsomes, which is not unusual for this
substrate (Potter et al., 1973). For RSV and BSA experiments,
approximately 0.8 nmol of acetaminophen per milligram of
protein were bound to the boiled blanks. For kidney medullary
microsomes, approximately 0.4 nmol/mg were bound to boiled
microsomes. These values remained constant throughout all experiments. A Student’s t test was performed on all data to
ensure significance.

Discussion

Our data indicate that PES metabolizes acetaminophen to
a reactive metabolite which covalently binds in vitro to protein,
and that the observed in vitro covalent binding of acetaminophen
to rabbit renal medullary microosomal protein is mediated
by PES, rather than by the cytochrome P-450 system. The
covalent binding of acetaminophen to RSV microsomal protein
in the presence of arachidonic acid, but inhibitable by indomethacin, supports the hypothesis that an electrophilic acet-
aminophen metabolite is generated during the oxidation of
arachidonic acid to prostaglandins. Our hypothesis is also sup-
ported by the recent report (Moldeus and Rahimtula, 1980)
demonstrating the formation of an acetaminophen-glutathione
conjugate during prostaglandin biosynthesis in RSV micro-
somes. Furthermore, these workers indicate that this glutathi-
one conjugate is similar to the conjugate formed by the mixed-
function oxidase activity of rat liver (Moldeus, 1978). Cumene
hydroperoxide, a substrate for the hydroperoxidase component
of PES, also supports acetaminophen binding and is not in-
hibited by the cylooxygenase inhibitor indomethacin. This
suggests that the prostaglandin hydroperoxidase-dependent
conversion of prostaglandin G2 to prostaglandin H2 is the key
step in the cooxidative metabolism of acetaminophen (fig. 5).
Stimulation of PES by acetaminophen, as measured by O2
uptake studies with RSV microsomes, further suggests that
acetaminophen is a cosubstrate for PES, as oxygen uptake is
stimulated in a dose-dependent manner. Stimulation of PES is
commonly observed by its chemical substrates (Egan et al.,

Our data from purified PES experiments provide strong
evidence that acetaminophen is metabolized by this enzyme
system. Purified PES supports a relatively large amount of
acetaminophen covalent binding to BSA added to the incuba-
tion system. The dependence of this binding on the presence
of arachidonic acid and its inhibition by indomethacin suggest a
cooxidative mechanism. Although a large amount of covalent
binding occurs in the absence of phenol, its addition stimulates
binding by approximately 2-fold. This appears to be related to
the ability of phenol to protect PES from self-inactivation
(Egan et al., 1976).

The human kidney has been reported to be a target organ of
acetaminophen-induced toxicity, which may occur with or with-
out concurrent hepatic injury (Boyer and Rouff, 1977; Mitchell
et al., 1977; Kincaid-Smith, 1980). There is also evidence that
chronic analgesic nephropathy originates in the medullary
region (Kincaid-Smith, 1980). This phenomenon has been studied
to a much lesser extent than that of hepatotoxicity resulting
from acetaminophen overdosage, the biochemical mechanism
of which is fairly well defined (Hinson, 1980). It is generally
accepted that acetaminophen is oxidized to a reactive inter-
mediate by the cytochrome P-450-dependent mixed-function
oxidase system (Mitchell et al., 1973a) present in the liver. It is
thought that this electrophilic intermediate then binds cova-
lently to cellular macromolecules, resulting primarily in centri-
lobular hepatic necrosis (Jollow et al., 1973). In rodents, it has
been demonstrated that the bound metabolite of acetaminophen
is covalently linked to individual amino acids (Jollow et al.,
1973), that hepatic glutathione levels correspond inversely
with covalently binding (Mitchell et al., 1973b) and that covalent
binding correlates directly with hepatotoxicity (Gillette,
1974). The structure of the reactive intermediate is still un-
known, but there is substantial evidence implicating N-acetyl-
p-benzoquinoneimine (Corcoran et al., 1980; Blair et al., 1980;
Fernando et al., 1980).

In addition to recent evidence that renal papillary necrosis
can be induced by acetaminophen (Kincaid-Smith, 1980), Dug-
gin and Mudge (1976) have performed animal studies in vivo
suggesting that acetaminophen accumulation occurs in the
medulla rather than the cortex. However, studies examining
the differential distribution of mixed-function oxidase activity
in the rabbit kidney indicate the presence of virtually no cyto-
chrome P-450 activity in the inner medulla and very little in
the outer medulla (Zenser et al., 1978). The levels of PES, on
the other hand, are relatively high in the inner medulla (Smith
and Wilkin, 1977). Other studies indicate that the covalent

**TABLE 4**

<table>
<thead>
<tr>
<th>Cumene hydroperoxide-dependent covalent binding of acetaminophen to rabbit renal medullary microsomes</th>
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</thead>
<tbody>
<tr>
<td>Acetaminophen Bound/mg Protein (nmol)</td>
</tr>
<tr>
<td>Complete system*</td>
</tr>
<tr>
<td>0.482 ± 0.006</td>
</tr>
<tr>
<td>− Cumene hydroperoxide</td>
</tr>
<tr>
<td>0.037 ± 0.023</td>
</tr>
<tr>
<td>+ Indomethacin</td>
</tr>
<tr>
<td>0.439 ± 0.025</td>
</tr>
</tbody>
</table>

* Complete system contains: 0.05 M phosphate buffer, pH 7.8; 2 mg of rabbit kidney medullary microsomes; 200 µM cumene hydroperoxide; 100 µM [7H] acetaminophen; and water to make 2 ml. Indomethacin = 200 µM.
binding of acetaminophen to kidney microsomes does not increase with prior 3-methylcholanthrene or phenobarbital treatment (Mudge et al., 1978). Both of these compounds induce mixed-function oxidase-mediated binding in the liver, however. Based on the above evidence and the observed PES-mediated metabolism of acetaminophen to a reactive intermediate, we suggest that possible nephrotoxicity of acetaminophen may be due, in part, to its metabolism in the inner medulla by PES.

Our data indicate that the covalent binding of acetaminophen to rabbit renal medullary microsomal protein is mediated by PES. Arachidonic acid, the major substrate for the cyclooxygenase component of PES, is required to support binding, and the cyclooxygenase inhibitor, indomethacin, inhibits binding. Some covalent binding occurs in the absence of arachidonic acid. With RSV and rabbit kidney medulla microsomes, this is most likely due to the endogenous arachidonic acid present in microsomes. Autooxidation of acetaminophen also occurs, accounting for the small amount of arachidonic acid-independent binding in the purified PES system. A prostaglandin hydroperoxide-dependent mechanism of cooxidation is again implicated by cumene hydroperoxide supporting the generation of a reactive acetaminophen intermediate. Indomethacin does not inhibit the cumene hydroperoxide dependent binding, as would be expected assuming prostaglandin hydroperoxide to be the enzyme responsible for cooxidation. Covalent binding was not supported by the addition of several different concentrations of NADPH to the incubation mixtures. Furthermore, Zenser et al. (1978) were unable to detect cytochrome P-450 in either the inner or outer medullary region of the rabbit kidney using a CO-reduced difference spectrum. This suggests that the cytochrome P-450-dependent mixed-function oxidase system plays a minor role in the conversion of acetaminophen to an intermediate capable of covalent binding in the rabbit renal medulla. The covalent binding to medullary protein is significantly reduced by the presence of reduced glutathione in the incubation mixture. Glutathione does not appear to alter the oxidation of chemicals by prostaglandin hydroperoxidase (Marnett et al., 1975).

The rate of acetaminophen metabolism by PES was relatively rapid. Covalent binding of acetaminophen to rabbit medullary microsomes had a K_m of 40 nM and was saturated at 100 nM. However, these constants can only be approximate, because enzyme activity was nonlinear throughout the period of analysis. These results are similar to those reported by Maldeus and Rahimtula (1980) for PES-dependent glutathione conjugate formation.

The nature of the reactive intermediate of acetaminophen that covalently binds to tissue and forms glutathione conjugates is not known. Electrochemical studies indicate that acetaminophen is similar in chemical nature to p-aminophenol (Miner and Kissenger, 1979), which undergoes one electron oxidation. Both p-aminophenol and p-phenyleinemide are oxidized by peroxidase to quinoneimines (Borei and Bjorklund, 1953; Frieden and Haieh, 1976; Mason, 1982). Recently, Blair et al. (1980) described the reaction of the acetaminophen quinoneimine with glutathione to form conjugates. Furthermore, Kissenger (1978) has shown that the quinoneimine intermediate can be generated electrochemically from acetaminophen and reacts with nucleophiles to produce sulfhydryl adducts that are similar to those formed by the mixed-function oxidase system. Thus, PES may oxidize acetaminophen by two sequential one-electron transfers to the N-acetyl-p-benzoquinoneimine, which is the presumed reactive intermediate. It is not known whether the second electron transfer is enzyme-dependent or not.

In conclusion, we propose that acetaminophen is cooxidized by PES in the renal medulla to a reactive intermediate which covalently binds to cellular macromolecules. The possibility that covalent binding correlates directly with the nephrotoxicity of acetaminophen, as in the liver, must be considered. Furthermore, our data substantiate the hypothesis that PES-mediated cooxidation may serve as an additional drug metabolism system in the renal medulla.

References


Mitchell, J. R., Jollow, D. J., Potter, W. Z., Davis, D. C., Gillette, J. R.

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