A Reiteration of the Equation Derived by Easson & Stedman (1936) and its Application to the Inhibition of Mitochondrial Energy-Linked Functions by the Aurovertins

PAUL E. LINNETT,* A. DAVID MITCHELL,* R. BRIAN BEECHY* and HAROLD BAUMt

*Shell Research Limited, Biosciences Laboratory, Sittingbourne Research Centre, Sittingbourne, Kent ME9 8AG, U.K., and tDepartment of Biochemistry, Chelsea College, University of London, Manresa Road, London SW3 6LX, U.K.

Applying the law of mass action to a system where an inhibitor binds to an enzyme and pursuing the arguments presented by Easson & Stedman (1936) and Straus & Goldstein (1943), the following equations can be derived:

\[ I_{\text{max}} = \frac{K_D}{E} + 0.5C \]  
\[ K_D = E(I_{\text{max}} - 0.5C) \]

where \( I_{\text{max}} \) is the relative concentration of inhibitor required to give half-maximal inhibition of the enzyme activity (e.g. in nmol of inhibitor/mg of protein), \( E \) is the enzyme concentration (e.g. in mg of protein/ml), \( C \) is the proportion of protein added that is catalytically active (e.g. in nmol of enzyme/mg of protein present) and \( K_D \) is the dissociation constant for the inhibitor–enzyme complex (with the indicated units, \( K_D \) will be expressed in micromolar terms).

For this simple treatment to be relevant, the following must apply:
(a) there is a single reversible binding site for the inhibitor;
(b) the binding of the inhibitor must reach equilibrium before the assay of enzyme activity;
(c) the binding of substrates have no effect on \( K_D \), i.e. the inhibition is non-competitive. Eqn. (1) shows that values of \( I_{\text{max}} \) for the inhibition of enzyme activity will vary with the concentration of enzyme protein in the assay (\( E \)) unless \( K_D \) is very small, i.e. when the inhibitor binds very tightly, or when \( E \) is large.

We have applied eqns. (1) and (2) to data that have been obtained in studies on the inhibition of the mitochondribral ATPase\dagger by the aurovertins. The structure of aurovertin B is given in Mulheirn et al. (1974). For the inhibition of the ATPase activity in submitochondrial particles by aurovertin B, the observed \( I_{\text{max}} \) values indeed vary with the enzyme concentration (Table 1). It can be seen, however, that the calculated \( K_D \) values vary little over the range of enzyme concentrations.

A body of data has been amassed which appears to demonstrate that ATP synthesis is considerably more sensitive to inhibition by aurovertins B and D than are reactions that rely on the hydrolysis of ATP (Lardy et al., 1964; Lenaz, 1965; Lee & Ernst, 1968; Roberton et al., 1968; Bertina et al., 1973). Table 2 shows that the values of \( I_{\text{max}} \) for the inhibition by aurovertin B of ADP-stimulated respiration, ATP-driven reduction of NAD+ by succinate and the soluble and membrane-bound ATPase activities vary over two orders of magnitude. The same data have been used to calculate \( K_D \) values by means of eqn. (2). The values obtained for \( K_D \) are remarkably constant (see Table 1).

Thus it appears that the affinity of the ATPase molecule for aurovertins B and D does not change significantly when the enzyme is operating in either its hydrolytic or its synthetic mode.

It is noteworthy that the values of \( K_D \) in Table 2 are similar to those obtained from (a) fluorescence-enhancement experiments with aurovertin D, where \( K_D = 0.02-0.52 \mu M \) (Chang & Peneisky, 1973; Van de Stadt et al., 1974), and (b) experiments to measure the binding of \(^3\text{H}\)aurovertin B to ox heart mitochondria and sub mitochondrial particles (A. D. Mitchell & P. E. Linnett, unpublished work).

Finally it should be pointed out that the aurovertins do not cause 100% inhibition of

\dagger Abbreviation: ATPase, adenosine triphosphatase (EC 3.6.1.3).
Table 1. Inhibition of ox heart submitochondrial-particle ATPase by aurovertin B measured at different enzyme concentrations

ATPase activity was measured by a coupled enzyme assay (Pullman et al., 1960). Aurovertin B was preincubated with submitochondrial particles in the assay mixture for 5 min at 30°C before the reaction was started with ATP. The dissociation constants were calculated from eqn. (2) (see the text), assuming that $C = 0.3$ nmol of ATPase/mg of protein.

<table>
<thead>
<tr>
<th>Concentration of submitochondrial particles in the assay (µg of protein/ml)</th>
<th>$I_{\text{max}}$ (nmol of aurovertin/mg of protein)</th>
<th>Calculated $K_D$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70.4</td>
<td>6</td>
<td>0.39</td>
</tr>
<tr>
<td>35.2</td>
<td>10</td>
<td>0.35</td>
</tr>
<tr>
<td>17.6</td>
<td>22</td>
<td>0.39</td>
</tr>
<tr>
<td>8.9</td>
<td>34</td>
<td>0.30</td>
</tr>
<tr>
<td>4.4</td>
<td>56</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Table 2. Inhibition by aurovertin B of energy-linked reactions of ox heart mitochondria

See the legend to Table 1. Values of $C = 0.2$, 0.3 and $2.8$ nmol of ATPase/mg of protein were assumed for mitochondria, submitochondrial particles and soluble ATPase respectively. ATP-driven reduction of NAD$^+$ by succinate and membrane-bound ATPase were in submitochondrial particles. ATP-driven ADP-stimulated reduction Membrane- of NAD$^+$ by bound Soluble ATPase ATPase

<table>
<thead>
<tr>
<th>$I_{\text{max}}$ (nmol of aurovertin/mg of protein)</th>
<th>ADP-stimulated respiration in mitochondria</th>
<th>ATP-driven reduction of NAD$^+$ by succinate</th>
<th>Membrane-bound ATPase</th>
<th>Soluble ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.19</td>
<td>0.65</td>
<td>34</td>
<td>30</td>
<td></td>
</tr>
</tbody>
</table>

$E$ (enzyme concentration in mg of protein/ml) | Calculated $K_D$ (µM) | 0.081 | 0.35 | 0.30 | 0.079 |

ATPase activity. In this case $I_{\text{max}}$ values are calculated for a maximum inhibition of around 85%. For some ATP-driven reactions the residual ATPase activity of the enzyme–inhibitor complex is not rate-limiting for the overall coupled process. In such cases (e.g. ATP-driven cation uptake in tightly coupled mitochondria), there may be a virtual insensitivity to aurovertins B and D (P. E. Linnett, A. D. Mitchell, R. B. Beechey & H. Baum, unpublished work). This is a separate source of variability and in no way invalidates the above analysis.

Straus, O. H. & Goldstein, A. (1943) J. Gen. Physiol. 26, 559–585