The next step in the purification involved degradation of the glycogen in the protein–
glycogen complex, in order to solubilize the various enzyme activities. This was achieved
for glycogen synthase by incubation of the complex with 0.5 m-potassium phosphate and
5 mM-AMP, which activates the endogenous phosphorylase that is present in the com-
pound (Nimmo et al., 1976), followed by extensive dialysis against 0.005 m-Tris/HCl/10% glycerol/15 mM-2-mercaptoethanol, pH 7.5. This procedure completely inactivated
phosphorylase phosphatase activity, the phosphate rather than the AMP being respon-
sible. Preincubation with Mn\(^{2+}\) partially restored the activity, but only to about 10% of
that in the protein–glycogen complex. A number of other cations (Mg\(^{2+}\), Ca\(^{2+}\), Zn\(^{2+}\),
Cu\(^{2+}\) and Fe\(^{3+}\)) had no significant effect, but Co\(^{2+}\) was considerably more effective than
Mn\(^{2+}\) in re-activating phosphorylase phosphatase activity (Table 1).

Preincubation of the protein–glycogen complex for 15 min with 1.0 mM-Mn\(^{2+}\) before
the treatment with 0.5 m-phosphate completely protected phosphorylase phosphatase
against the loss of activity. However, preincubation with Co\(^{2+}\) at this stage surprisingly
decreased the phosphorylase phosphatase activity by between 60 and 90%. Preincubation
of muscle extracts with Co\(^{2+}\) similarly inactivated phosphorylase phosphatase by
70–90%.

The results presented above suggest that purification of phosphorylase phosphatase in
the presence of EDTA or treatment with high concentrations of phosphate causes the
enzyme to lose a cofactor. The only molecules that have been found to reverse this loss
of activity are the bivalent cations Mn\(^{2+}\) and Co\(^{2+}\), and the only molecule capable of
protecting the enzyme against inactivation by phosphate is Mn\(^{2+}\). These results suggest
that protein phosphatase-III may either be a manganese or cobalt metalloenzyme in vivo.

However, the enzyme that was re-activated by Co\(^{2+}\) was inhibited by further pre-
incubation with Mn\(^{2+}\) (Table 1), whereas Mn\(^{2+}\) had no effect on the activity of the muscle
extract or the protein–glycogen complex. In contrast, when the enzyme that had been
re-activated by Mn\(^{2+}\) was further preincubated with Co\(^{2+}\), the activity was inhibited
70–90% (Table 1), which was similar to that observed when the muscle extract or the
protein–glycogen complex was preincubated with Co\(^{2+}\). These observations suggest that
the enzyme in muscle extracts is likely to contain Mn\(^{2+}\) rather than Co\(^{2+}\).

The ability of Mn\(^{2+}\) to protect the enzyme in the protein–glycogen complex against
inactivation by high concentrations of phosphate, even though this enzyme should
already contain this cofactor, as it is fully active, suggests that Mn\(^{2+}\) must bind to at
least two sites on the enzyme. The potent inhibition of the Mn\(^{2+}\)-activated enzyme by
Co\(^{2+}\) and the strong activation of the presumed apoenzyme by Co\(^{2+}\) also show that there
are at least two bivalent-cation-binding sites on the enzyme.

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Purification and Properties of an Alcohol Dehydrogenase from the Liver
and Intestinal Caecum of Rainbow Trout (Salmo gairdnerii)

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The prominence of wax esters in the natural diets of numerous marine fish such as salmon
and herring is well established (Benson et al., 1972; Sargent et al., 1976). Dietary wax
esters appear to be hydrolysed in fish intestines to their constituent fatty acids and fatty
alcohols (Patton et al., 1975), which are then absorbed and converted into triacyl-
glycerols (Sand et al., 1973). Alcohol dehydrogenase (EC 1.1.1.1) is present in the livers
of many animals, including fish, and has a broad substrate specificity (Sund & Theorell, 1963). We establish here that alcohol dehydrogenase is present in both the livers and intestinal caeca of a salmonid fish, the rainbow trout *Salmo gairdnerii*, and describe some properties of the enzyme, including its ability to oxidize hexadecanol.

Rainbow trout (150–300g) were fed on a standard diet of trout pellets. The fish were killed by a blow on the head and their livers and intestinal caeca homogenized at 2°C in 3 vol. of 10mM-Tris/HCl, pH 8.0, containing 1mM-2-mercaptoethanol. Alcohol dehydrogenase activities were purified from the homogenates at 2–4°C by: (1) centrifuging at $6.0 \times 10^6$g$_{av}$-min and retaining the supernatants; (2) passing through DEAE-cellulose at pH 8.0 and retaining the effluent; (3) affinity chromatography on Blue Sepharose CL-6B in 0.1M-sodium phosphate, pH 7.0, containing 1mM-2-mercaptoethanol, where the enzyme was eluted with 5mM-NAD$^+$ in the same buffer. Enzymic activity was routinely assayed spectrophotometrically at 23°C in 0.1M-sodium pyrophosphate, pH 9.0, 1mM-2-mercaptoethanol, 20mM-ethanol and 1mM-NAD$^+$. Protein was determined by the method of Lowry et al. (1951).

The final preparations of trout alcohol dehydrogenase had specific activities of 1.6 (liver) and 1.4 (caecum) pmol of ethanol oxidized/min per mg of protein, representing purifications of 150-fold and 900-fold respectively. For comparison, crystallized horse liver alcohol dehydrogenase (purchased from Sigma Chemical Corp.) had a specific activity of 1.3 pmol/min per mg of protein. Both trout enzymes moved as zones, approx. mol.wt. 80000, on columns of Sephadex G-200 developed in 0.1M-potassium phosphate buffer, pH 7.0, containing 1mM-2-mercaptoethanol. Both enzymes migrated as zones, approx. mol.wt. 40000, when analysed by electrophoresis on polyacrylamide gels containing 0.1% sodium dodecyl sulphate. Histochemical assays after conventional electrophoresis on polyacrylamide gels showed that the liver enzyme had one major and two minor zones of ethanol dehydrogenase activity, whereas the caecal enzyme had a single zone of activity. Other properties of the enzymes, including the maximal rates at which they oxidized water-soluble alcohols, are summarized in Table 1, where data for horse liver alcohol dehydrogenase are reproduced for comparison. We conclude that the trout enzymes are structurally similar to horse liver alcohol dehydrogenase, but differ from it in terms of substrate specificity.

### Table 1. Some properties of the alcohol dehydrogenase from trout liver, trout intestinal caeca and horse liver

Data quoted for horse liver alcohol dehydrogenase, except for the value for hexadecanol, were compiled from Sund & Theorell (1963) and Bränden et al. (1975) with some recalculation. All other data were determined in the present work by methods detailed in the text. Kinetic constants for the trout enzymes were determined by the method of Atkins et al. (1978). Reaction rates for trout enzymes were measured at saturating substrate concentrations, except for hexadecanol. Rates for hexadecanol for both trout and horse liver enzymes were determined at a concentration of 4µM.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Trout liver</th>
<th>Trout caecum</th>
<th>Horse liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>5</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Propanol</td>
<td>85</td>
<td>95</td>
<td>24</td>
</tr>
<tr>
<td>Butanol</td>
<td>70</td>
<td>74</td>
<td>79</td>
</tr>
<tr>
<td>Pentanol</td>
<td>44</td>
<td>36</td>
<td>22</td>
</tr>
<tr>
<td>Butan-2-ol</td>
<td>5</td>
<td>7</td>
<td>24</td>
</tr>
<tr>
<td>Hexadecanol</td>
<td>0.020</td>
<td>0.016</td>
<td>0.0007</td>
</tr>
</tbody>
</table>
To test whether fatty alcohol was a substrate for trout alcohol dehydrogenase we incubated the enzymes, purified to the DEAE-cellulose stage, in the standard assay containing 1.7 mm-NAD$^+$ and [9,10-$^3$H$_2$]hexadecanol added in 10 µl of acetone to give 2 µCi and 4 nmol/ml. The reaction was stopped by extracting total lipid. Fatty alcohol and fatty acid were separated on thin layers of silicic acid in light petroleum (b.p. 40–60°C), diethyl ether/acetic acid (70:30:1, by vol.) and radioassayed by liquid-scintillation spectrometry. The results (Fig. 1) showed that both liver and caecal enzyme preparations catalysed an NAD$^+$-dependent oxidation of fatty alcohol to fatty acid at initial rates of 0.4 (liver) and 0.16 (caecum) nmol/h per mg of protein. In these experiments disappearance of radioactivity from the hexadecanol zone was closely paralleled by appearance of radioactivity in the hexadecanoic acid zone, indicating that the enzyme preparations contained substantial aldehyde dehydrogenase activity. Horse liver alcohol dehydrogenase also oxidized hexadecanol, but at much lower rate, relative to ethanol, than the trout enzymes (Table 1).

These results demonstrate that intestinal and liver alcohol dehydrogenase can oxidize fatty alcohol and support the proposition that these enzymes may be involved in the metabolism of dietary wax esters in salmonid fish.

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Bräden, C.-I., Jörnvall, H., Eklund, H. & Furugen, B. (1975) Enzymes 3rd Ed. 11, 103–190