Uncoupling Agents: Arsenical Analogues of Pyrophosphate and their Compounds

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Arsenate has long been used as an analogue of orthophosphate in biochemical reactions, especially in the enzymic oxidation of glyceral 3-phosphate (Needham & Pillai, 1937; Warburg & Christian, 1939) to change the overall reaction from eqn. (1) to eqn. (2).

\[
R-\text{CHO} + \text{Pi} + \text{NAD}^+ \rightarrow R-\text{CO-O-PO(OH)_{2}} + \text{NADH} + \text{H}^+ \quad (1)
\]

\[
R-\text{CHO} + \text{H}_2\text{O} + \text{NAD}^+ \rightarrow R-\text{CO}_{2}\text{H} + \text{NADH} + \text{H}^+ \quad (2)
\]

Arsenate does this because the enzyme accepts it as orthophosphate, but the first product breaks down because of the instability of esters and anhydrides of arsenate (cf. Braunstein, 1931).

This use of arsenate is limited because most of the biochemical reactions that liberate orthophosphate, in contrast with those that liberate pyrophosphate, are not freely reversible. Arsenical analogues of pyrophosphate may therefore be expected to undergo similar reactions with a wide range of enzymes. They should change a reaction of the type:

\[
\text{NTP} + \text{X-H} \rightarrow \text{N-O-PO(OH)-X} + \text{PP_i}
\]

(\text{where N is a nucleoside}) to

\[
\text{NTP} + \text{H}_2\text{O} \rightarrow \text{NMP} + \text{PP_i}
\]

or

\[
\text{N-O-PO(OH)-X} + \text{H}_2\text{O} \rightarrow \text{NMP} + \text{X-H}
\]

according to whether NTP and X-H or N-O-PO(OH)-X are added.

Myers et al. (1963) showed the extensive biochemical use that could be made of methylenediphosphonic acid, (HO)\textsubscript{2}PO-CH\textsubscript{2}-PO(OH)\textsubscript{2}, and its compounds as pyrophosphate analogues (for a review, see Yount, 1975). The substitution of CH\textsubscript{2} for O in pyrophosphate allows the further substitution of one or both phosphorus atoms by arsenic.

We have therefore made arsonomethylphosphonic acid, (HO)\textsubscript{2}AsO-CH\textsubscript{2}-PO(OH)\textsubscript{2}, by the Meyer (1883) reaction, which involves treating an alkyl halide, in our case chloromethylphosphonic acid, with arsenite in alkali. Methyleneiarsonic acid, (HO)\textsubscript{2}AsO-CH\textsubscript{2}AsO(OH)\textsubscript{2}, has been made by using the Cadet (1760) reaction by both Popp (1949) and Titov & Levin (1953). These last authors treated As\textsubscript{2}O\textsubscript{3} with acetic anhydride in the presence of potassium acetate, and oxidized the CH\textsubscript{2}(As=O)\textsubscript{2} produced with H\textsubscript{2}O\textsubscript{2}; we followed their procedure and simplified the isolation of the methyleneiarsonic acid.

We are exploring the biochemical use of these compounds in two main ways. One is to study their effects in biochemical reactions of pyrophosphate. The other is to prepare esters of arsonomethylphosphonic acid as analogues of natural diphosphates, e.g. Ado({}^{-}5')-O-PO(OH)-CH\textsubscript{2}-AsO(OH)\textsubscript{2} as an analogue of ADP. Any system that generates ATP from ADP and uses this compound as ADP should be converted into an adenosine triphosphatase ('uncoupled') by its presence.

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Brown adipose tissue occurs in hibernators, cold-adapted and newborn mammals and is specialized for thermogenesis under the direct control of the sympathetic nervous system (Joel, 1965; Himms-Hagen, 1970). Heat is produced by the highly exothermic process of fatty acid oxidation. The thermogenic capacity of the tissue is therefore determined by the respiratory rate. In most tissues the rate of respiration is controlled and linked to the cellular demand for ATP synthesis. Brown-adipose-tissue mitochondria have developed a special mechanism for obtaining maximal uncontrolled rates of respiration independent of ATP synthesis. This is achieved by the presence of a unique pathway through which protons leak back into the matrix, thus preventing the build up of a proton electrochemical gradient \((\Delta \mu_{H^+})\) across the mitochondrial inner membrane of sufficient magnitude to induce respiratory control (Nicholls & Lindberg, 1973; Nicholls \textit{et al.}, 1974; Nicholls, 1974a,b).

A mechanism must therefore exist to inhibit this pathway in the absence of sympathetic stimulation when thermogenesis is not required. This can be achieved \textit{in vitro} by the addition of exogenous purine nucleotide di- or tri-phosphates to the isolated mitochondria (Hohorst & Rafael, 1968; Rafael \textit{et al.}, 1969; Pederson, 1970; Nicholls, 1976). The nucleotides bind to unique sites on the outer face of the inner membrane (without covalent modification), which are independent of the adenine nucleotide translocase, and are absent from rat liver mitochondria. The affinities, specificities, pH-dependence and capacity (0.7 nmol/mg) of binding site closely correspond to the conditions required for the inhibition of the ion-conductance pathway (Nicholls, 1976). This nucleotide-sensitive proton-conductance pathway thus appears to be responsible for the unique bioenergetic behaviour of brown-adipose-tissue mitochondria.

If this binding site truly controls the proton-conductance pathway \textit{in vivo}, the inhibition must be freely reversible, ADP has a high affinity for the binding site, and therefore strongly inhibits the ion-conductance pathway, enabling a high \(\Delta \mu_{H^+}\) to develop across the inner membrane (Table 1). Apyrase hydrolyses ADP to AMP, which does not inhibit the conductance. Mitochondria were incubated at 23°C as outlined in Fig. 1; \(\Delta \mu_{H^+}\) was measured before and after ADP addition and then at regular intervals after the addition of apyrase (Nicholls, 1974a,b). The high \(\Delta \mu_{H^+}\) induced by the presence of ADP was maintained for approx. 4 min after the addition of apyrase and then fell steadily back towards its initial value. Mitochondria incubated with ADP for the same length of time in the absence of apyrase showed no fall in \(\Delta \mu_{H^+}\). Control experiments with \(\text{[H]}\)ADP confirmed that hydrolysis of ADP was largely complete within the time-course of the experiment. Therefore the nucleotide inhibition of proton conductance appears to be freely reversible.

Since the physiological inhibitor is unknown, it is important to establish the structural specificity of the binding site.

The capacity of a wide variety of analogues, to bind and inhibit proton conductance (measured by their ability to elevate \(\Delta \mu_{H^+}\)) was compared with that of GDP (see Table 1). The presence of both the \(\alpha\)- and \(\beta\)-phosphate groups with their normal oxygen linkage, the absence of any bulky substitution on the \(\beta\)- or \(\gamma\)-phosphate and the presence of...