- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
 Tierney, D. F., Clements, J. A. & Trahan, H. J. (1967). *Amer. J. Physiol.* **213**, 671.

Influence of Metal Ions on the Orthophosphatase and Inorganic Pyrophosphatase Activities of Human Alkaline Phosphatase

By D. W. Moss. (*Department of Clinical Chemistry, University of Edinburgh, The Royal Infirmary, Edinburgh*)

It is now recognized that mammalian alkaline phosphatases (EC 3.1.3.1) are able to hydrolyse both pyrophosphate and orthophosphate substrates (Cox & Griffin, 1965; Moss, Eaton, Smith & Whitby, 1967; Fernley & Walker, 1967), and it seems probable that a single type of active centre is responsible for activity towards both classes of substrate (Eaton & Moss, 1967a). However, Mg^{2+} ions have a differential effect on the two activities of alkaline phosphatase, activating the hydrolysis of orthophosphates but inhibiting that of pyrophosphates (Fernley & Walker, 1967; Eaton & Moss, 1967a,b). The influence of Zn^{2+} and Mg^{2+} ions on the activities of purified human intestinal alkaline phosphatase (Moss *et al.* 1967) has now been examined in more detail, and the results indicate that the differential effects of these ions are consistent with the suggestion that both catalytic activities are properties of a single type of active centre.

Both the inorganic pyrophosphatase and orthophosphatase (substrate, *p*-nitrophenyl phosphate) activities of the enzyme were measured at pH 9.0 in tris-HCl buffer; at this pH both substrates are hydrolysed at measurable rates over a wide range of concentration. Addition of Zn^{2+} ions to the pyrophosphatase assay system almost completely inhibits release of inorganic phosphate when the concentration of Zn^{2+} is greater than that of the substrate, inorganic pyrophosphate. As the concentration of pyrophosphate is increased above that of Zn^{2+} , competitive inhibition of the pyrophosphatase activity is seen. These observations can be explained by assuming that, when Zn^{2+} ions are in excess, all the substrate is in the form of a Zn^{2+} -pyrophosphate complex which is not broken down by the enzyme, or is only slowly attacked. As the substrate concentration is increased free pyrophosphate appears and is hydrolysed, but the Zn^{2+} -pyrophosphate complex is a competitive inhibitor of this hydrolysis. The Zn^{2+} -pyrophosphate complex is also a competitive inhibitor of the orthophosphatase activity when Zn^{2+} and pyrophosphate are added to this assay system in proportions calculated to produce the complex.

A similar explanation seems to be valid for the effect of Mg^{2+} ions on the pyrophosphatase activity of alkaline phosphatase. However, the inhibition of pyrophosphate hydrolysis by the Mg^{2+} -pyrophosphate complex is less marked than that by the Zn^{2+} complex, and the stoichiometry of the interactions of the two metal ions with pyrophosphate appears to be different.

- Cox, R. P. & Griffin, M. J. (1965). *Lancet*, ii, 1018.
 Eaton, R. H. & Moss, D. W. (1967a). *Biochem. J.* **102**, 917.
 Eaton, R. H. & Moss, D. W. (1967b). *Biochem. J.* **105**, 1307.
 Fernley, H. N. & Walker, P. G. (1967). *Biochem. J.* **104**, 1011.
 Moss, D. W., Eaton, R. H., Smith, J. K. & Whitby, L. G. (1967). *Biochem. J.* **102**, 53.

The Role of Endogenous Cations in the Na^+ - K^+ - Mg^{2+} -Effected Adenosine Triphosphatase of Cerebral Microsomes

By P. S. G. GOLDFARB and R. RODNIGHT. (*Department of Biochemistry, Institute of Psychiatry, British Postgraduate Medical Federation, University of London, London, S.E. 5*)

Hydrolysis of [^{32}P]ATP by cerebral microsomes is associated with a Na^+ -dependent and K^+ -inhibited binding of ^{32}P to a protein component in the membrane (Rodnight, Hems & Lavin, 1966, and references cited therein). Assuming that the Na^+ - K^+ - Mg^{2+} -effected ATPase* proceeds by a multistage reaction, the action of K^+ can be considered as one of increasing the turnover of a phosphorylated intermediate, this resulting in a lower steady-state content of intermediate when ATP is limited. With $2.5\mu M$ -ATP and twice-washed microsomes, however, appreciable Na^+ -dependent hydrolysis of ATP occurs in the absence of added K^+ and Mg^{2+} , the steady-state content of bound ^{32}P declining to zero as hydrolysis approaches completion. This suggests that sufficient endogenous K^+ and Mg^{2+} are present to permit the Na^+ - K^+ - Mg^{2+} -effected ATPase to hydrolyse very low concentrations of ATP.

The above observations were made with a microsomal preparation prepared by a modification of an earlier method (Rodnight & Lavin, 1966) involving several centrifugal washes of the crude microsomes to remove free cations. The preparation contained ($m\mu$ moles/mg. of protein): Mg^{2+} , 20 ± 2 ; Na^+ , 90 ± 20 ; K^+ , 27 ± 3 . By further washing with EDTA-containing buffers these levels have now been decreased to: Mg^{2+} , 3 ± 1 ; Na^+ , 24 ± 12 ; K^+ , 7 ± 2 . The exhaustively washed preparations showed a definitive requirement for Mg^{2+} for full phosphorylation, but turnover of bound ^{32}P still continued, presumably due to the residual bound

* Abbreviation: ATPase, adenosine triphosphatase.

K⁺. However, the turnover rate and ATP hydrolysis was slowed and the steady state content of bound ³²P prolonged; readdition of very low concentrations of K⁺ (e.g. 2 μM) restored full activity.

Attempts to lower the residual K⁺ still further have been largely unsuccessful. By washing with 20 mM-NaCl or 10 mM-Na₂ATP (± Mg²⁺), concentrations of K⁺ of approx. 3 mμmoles/mg. of protein have been achieved, but the Na⁺-dependent ATPase activity of such preparations was not decreased. Gentle disruptive procedures (deoxycholate, low concentrations of urea or guanidine hydrochloride) and washing at pH 5 or 9 also failed to release bound K⁺. These results support the hypothesis that the Na⁺-dependent hydrolysis of micromolar concentrations of ATP is due to the activity of the Na⁺-K⁺-Mg²⁺-effected ATPase to which intrinsic bound K⁺ in the membrane is accessible.

We are grateful to the U.S. Public Health Service for Grant no. NB 05502-05 for support, and to the Medical Research Council for a scholarship to P.S.G.G.

Rodnight, R., Hems, D. A. & Lavin, B. E. (1966). *Biochem. J.* **101**, 502.

Rodnight, R. & Lavin, B. E. (1966). *Biochem. J.* **101**, 495.

Properties and Characterization of Haem Hydroxylase in Liver and Kidney Microsomes of Rat and Mouse

By O. NAKAJIMA and C. H. GRAY. (*Department of Chemical Pathology, King's College Hospital Medical School, London, S.E. 5*)

Pyridine haemichromogen is converted aerobically into a green pigment after incubation at 37° for 30 min. with microsomes from liver and kidney of rat or mouse. The properties of the microsomal enzyme in liver and kidney resemble those of haem α-methenyl oxygenase in the liver supernatant fraction from ox and guinea pig (Nakajima, Takemura, Nakajima & Yamaoka, 1963), but the pH optimum of the microsomal enzyme is 9.0 in either 0.5 M-tris-HCl buffer or 0.5 M-borate buffer. The activity was destroyed on heating to 100° for 5 min. but was not lost during storage at -20° for 2 weeks; in air the activity was completely lost at 4° within 3 days. The microsomal enzyme is thus more stable at -20° than haem α-methenyl oxygenase in liver supernatant. Metal-binding reagents such as o-phenanthroline and αα'-bipyridyl, and the thiol inhibitor p-chloromercuribenzoate inhibited the microsomal enzyme, as the supernatant enzyme; catalase was without effect, suggesting that no hydrogen peroxide formation occurred in this process. The enzymic activity in

the microsomal fraction was lost on dialysis against 0.1 M-sodium phosphate buffer, pH 7.5, for 3 hr., and was restored by 0.1 mM-Fe²⁺ and 0.1 mM-NADPH.

The green product formed in the reaction mixture was extracted with chloroform and washed with 0.01 N-HCl and water. The pigment in the chloroform layer showed four absorption maxima at 660, 560, 530 and 404 nm. This absorption spectrum is different from that of formylbiliverdin-iron complex, the final product of haem α-methenyl oxygenase. Schwartz (1967) suggested that the main sites of the formation of early-labelled bile pigment are liver and kidney, and the microsomal enzyme in animal liver and kidney may thus contribute to the formation of this pigment *in vivo*. The reaction mechanism of the enzyme will be discussed in relation to that of the chemical degradation of haem.

The relationship of this enzyme to the microsomal haem oxygenase described by Tenhunen, Marver & Schmid (1968) will be discussed.

Nakajima, H., Takemura, T., Nakajima, O. & Yamaoka, K. (1963). *J. biol. Chem.* **238**, 3784.

Schwartz, S. (1967). *Bilirubin Metabolism*, p. 15. Oxford: Blackwells Scientific Publications.

Tenhunen, R., Marver, H. S. & Schmid, R. (1968). *Proc. nat. Acad. Sci., Wash.* (in the Press).

Studies on Haem-Splitting Enzyme in Mitochondria of Rat and Mouse Spleen

By C. H. GRAY and O. NAKAJIMA. (*Department of Chemical Pathology, King's College Hospital Medical School, London, S.E. 5*)

The formation of bile pigment from haemin by homogenates prepared from liver, kidney, spleen, intestine, lung, heart and brain of mouse or rat has been investigated. After 2 hr. at 37° 0.1 mM-haemin at pH 8.6, an absorption maximum of 656 nm. in acid solution appeared with the spleen homogenate; weak activity was observed with homogenates from lung, heart and brain, but no activity was found with liver and kidney. The activity was higher in 0.1 M-phosphate buffer, pH 7.5, than in 0.25 M-sucrose solution, pH 7.5, or in 0.14 M-KCl solution at the same pH. The activity of the spleen homogenate was destroyed on heating at 100° for 10 min. Activity was present mainly in the mitochondrial fraction and to a smaller extent in the supernatant fraction. The pH optimum of the reaction with the mitochondrial fraction was between 8.0 and 8.5, at which the reaction proceeded linearly during the first 60 min. The reaction occurred in aerobic or anaerobic conditions. The