Action of Purified Alkaline Phosphatases from Human Liver and Small Intestine on Nucleoside Phosphates

By R. HELEN EATON and D. W. MOSS. (*University Department of Clinical Chemistry, The Royal Infirmary, Edinburgh*, 3)

Alkaline phosphatases (EC 3.1.3.1) from human liver and small intestine hydrolyse inorganic pyrophosphate, and the orthophosphatase and pyrophosphatase activities are not separated during extensive purification (Moss, Eaton, Smith & Whitby, 1967). The action of these phosphatases on ATP, ADP and AMP has now been examined, after purification which increased the specific activities of the enzymes by factors of 340 and 135 respectively.

Both enzyme preparations hydrolysed each of the three nucleotides, liberating inorganic phosphate. At 2mM substrate concentrations, the pH optima were in the region pH 9–9·5, and with the di- and triphosphates there was marked inhibition by excess substrate. With liver enzyme, similar values for K_m (about 2mM) were obtained with AMP, ADP and ATP as substrates, and also for intestinal phosphatase acting on AMP; however, lower K_m values (about 0·6–0·8mM) were observed for the hydrolysis of ADP and ATP by the latter enzyme. These measurements were made without addition of Mg^{2+}.

The relative rates of hydrolysis of the nucleotides, measured by release of inorganic phosphate at 2mM concentration of each substrate, without added Mg^{2+}, differed considerably for the enzymes from the two tissues: taking hydrolysis of *p*-nitrophenyl phosphate (*p*NPP) as 100, the velocities for liver phosphatase were AMP = 75, ADP = 50, and ATP = 20, and for intestinal phosphatase AMP = 125, ADP = 160 and ATP = 150. Addition of Mg^{2+} (10mM) markedly altered this pattern of substrate specificity. Under these conditions the relative velocities became, for the liver phosphatase, AMP = 130, ADP = 45 and ATP = 10, and for the intestinal enzyme, AMP = 155, ADP = 60 and ATP = 20, again referred to hydrolysis of *p*NPP (without Mg^{2+}) as 100. The inhibition by added Mg^{2+} of activity towards the pyrophosphate substrates, ADP and ATP, which is particularly marked in the case of intestinal phosphatase, resembles the effect of this ion on inorganic pyrophosphatase activity (Eaton & Moss, 1967). As with inorganic pyrophosphatase activity, intestinal phosphatase hydrolyses the organic pyrophosphates more readily than liver phosphatase.

Mixed-substrate experiments demonstrated inhibition by one class of substrate of hydrolysis of the other and thus support the hypothesis that the two types of activity are properties of a single enzyme in each preparation. Fernley & Walker (1966) have shown that calf-intestinal alkaline phosphatase will hydrolyse inorganic pyrophosphate and ATP, and have noted inhibition by Mg^{2+} of this activity.

We thank the Medical Research Council for financial support.

Eaton, R. H. & Moss, D. W. (1967). *Biochem. J.* **102**, 917.
Fernley, H. N. & Walker, P. G. (1966). *Biochem. J.* **99**, 39P.
Moss, D. W., Eaton, R. H., Smith, J. K. & Whitby, L. G. (1967). *Biochem. J.* **102**, 53.

Electron-Donor Requirements in the Hydroxylation of *p*-Coumaric Acid

By P. F. T. VAUGHAN and V. S. BUTT. (*Department of Botany, South Parks Road, Oxford*)

An enzyme preparation catalysing the hydroxylation of *p*-coumaric acid to caffeic acid has been extracted from spinach leaves and purified 200-fold by successive ammonium sulphate fractionation, thermal treatment, dialysis and chromatography on DEAE-cellulose columns. Hydroxylation activity was determined by caffeic acid production (Hoepfner, 1932) when *p*-coumaric acid was incubated in air at 30° with a reductant in citrate–phosphate buffer (0·05M, pH 5·3) with added ammonium sulphate (0·5M). This activity was accompanied at all stages of purification by a constant ratio of catecholase (EC 1.10.3.1) activity, from which it appears inseparable. The preparation contains copper and is inhibited by diethyldithiocarbamate (0·1mM).

Estimation of *p*-coumaric acid utilization (modified method of Lugg, 1937) showed an equivalent production of caffeic acid. Ascorbate was required as an electron donor both for hydroxylation and subsequent reduction of the quinone, the reaction being terminated with exhaustion of the ascorbate supply. A comparison between oxygen consumption and caffeic acid production at this stage showed one molecule of ascorbate utilized for the hydroxylation of each molecule of *p*-coumaric acid, suggesting that this enzyme acts as a mixed-function oxidase (Mason, 1957).

Caffeic acid could not act as a major electron donor, but tetrahydrofolic acid, reduced NAD and reduced NADP could substitute for ascorbate. The most effective donor so far found has been 6,7-dimethyl-5,6,7,8-tetrahydropteridine (DMTP), which stimulates hydroxylation to three times the rate with ascorbate and shows a direct equivalence between caffeic acid production and donor supply. Reduced NAD is also more effective than ascorbate, but acts as a reductant for the quinone as well. With

catalytic quantities of DMTP, ascorbate and reduced NAD can sustain the reaction at the maximum rate with DMTP alone. At saturating concentrations of DMTP and reduced NAD supplied together, caffeic acid is produced at the maximum rate observed with DMTP alone.

It is suggested that reduced NAD and DMTP act at the same site on the enzyme. The natural donor in the hydroxylation may be DMTP, reducible by reduced NAD, perhaps by the catalytic action of the enzyme. In these respects, the hydroxylation of *p*-coumaric acid is similar to that of cinnamic acid and phenylalanine (Nair & Vining, 1965*a*,*b*) in plants, for which no participation of metallic ions has been reported, and phenylalanine hydroxylase (Kaufman, 1962) and several phenolase systems (Pomerantz, 1966; Nagatsu, Levitt & Udenfriend, 1964) in animals.

One of us, P.F.T.V., wishes to gratefully acknowledge the receipt of a Scientific Research Council Studentship.

Hoepfner, W. (1932). *Chemikerzeitung*, **56**, 991.
Kaufman, S. (1962). In *Oxygenases*, p. 129. Ed. by Hayaishi, O. New York: Academic Press.
Lugg, J. W. H. (1937). *Biochem. J.* **31**, 1422.
Mason, H. S. (1957). *Advanc. Enzymol.* **19**, 79.
Nagatsu, T., Levitt, M. & Udenfriend, S. (1964). *J. biol. Chem.* **239**, 2910.
Nair, P. M. & Vining, L. C. (1965*a*). *Phytochem.* **4**, 161.
Nair, P. M. & Vining, L. C. (1965*b*). *Phytochem.* **4**, 401.
Pomerantz, S. H. (1966). *J. biol. Chem.* **241**, 161.

Effects of Hydroxyurea Treatment on Nucleic Acid Synthesis in Cultures of Pokeweed Mitogen-Stimulated Human Lymphocytes

By NORMA E. TOPPING, J. KIERSKI* and E. H. COOPER. (*Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W.* 3)

Hydroxyurea, effective in the chemotherapy of several forms of cancer (Thurman, 1964) causes reversible inhibition of DNA synthesis in various biological systems but has little effect on the synthesis of RNA (Young & Hodas, 1964; Yarbro, Kennedy & Barnum, 1965; Rosenkranz, Garrö, Levy & Carr, 1966). Yarbro *et al.* (1965) suggested that inhibition of RNA synthesis may result solely from the inhibition of DNA synthesis.

Epstein & Stohlman (1964) showed that 24hr. after the addition of phytohaemagglutinin to a suspension of human lymphocytes, 55% of the cells were synthesizing RNA whereas DNA synthesis was not appreciable until between 24 and 48hr. Such cultures are useful in the investigation of the effects of drugs on RNA synthesis in the absence of DNA synthesis, and on gene de-repression, DNA synthesis and chromosomes. Pokeweed mitogen causes much less leucoagglutination than phytohaemagglutinin and its use enables counts of total leucocyte number to be made.

Pokeweed mitogen-stimulated lymphocyte cultures (3ml.) were set up as previously described (Chalmers, Cooper, Evans & Topping, 1967), the same washing procedure being used for removal of drugs where necessary. For assessment of DNA or RNA synthesis [^{3}H]thymidine (2·9c/mM) or [^{3}H-uridine (2·7c/mM) was added (2μc/ml.) for 1hr., after which tritium incorporation into the nucleic acids was measured by scintillation counting as previously described (Chalmers *et al.* 1967).

A 4hr. exposure of a 48hr. culture to hydroxyurea sufficient to cause a large inhibition of DNA synthesis, caused only a small inhibition of RNA synthesis. Both effects occurred rapidly. RNA synthesis in a 24hr. culture was also inhibited by hydroxyurea though less so. The hydroxyurea treatment did not cause a decline in cell number during the experimental period. The presence of hydroxyurea during a 30min. exposure to stimulant and for 1hr. previously, had no effect on subsequent RNA and DNA synthesis, but the presence of hydroxyurea during the first 24hr. of culture caused decreased DNA synthesis at 48hr. probably due to the inhibition of RNA synthesis.

Chalmers, D. G., Cooper, E. H., Evans, C. & Topping, N. E. (1967). *Int. Arch. Allergy*, (in the Press).
Epstein, L. B. & Stohlman, F. Jr. (1964). *Blood*, **24**, 69.
Rosenkranz, H. S., Garro, A. J., Levy, J. A. & Carr, H. S. (1966). *Biochim. biophys. Acta*, **114**, 501.
Thurman, W. (1964). *Cancer Chemother. Rep.* **40**.
Yarbro, J. W., Kennedy, B. J. & Barnum, C. P. (1965). *Proc. Nat. Acad. Sci. Wash.*, **53**, 1033.
Young, C. W. & Hodas, S. (1964). *Blood*, **146**, 1172.

* Visiting worker from II Klinika Potoznicra Akademii Medycznej, Warsaw, Poland.

Effect of Phytohaemagglutinin on Lymphocyte Ribonucleic Acid synthesis

By J. E. KAY. (*Department of Biochemistry, University of Cambridge*)

When human lymphocytes are incubated *in vitro* with phytohaemagglutinin (PHA) they transform into large, rapidly dividing, lymphoblasts (Robbins, 1964). The rate of incorporation of [^{3}H]uridine into RNA increases rapidly after the addition of PHA (Epstein & Stohlman, 1964) and an increase in the rate of protein synthesis is found after 2–3hr. (Kay, 1966). The RNA synthesized by unstimulated lymphocytes is predominantly non-ribosomal, but after incubation with PHA for 5–6hr. there is a